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Serum concentrations of persistent organic pollutants mixture during pregnancy and anogenital distance in 8-year-old children from the INMA-Asturias cohort

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ABSTRACT

Background: During pregnancy, women are commonly exposed to several endocrine-disrupting chemicals, including persistent organic pollutants (POPs). These compounds can transfer to the fetus through the placenta. Prenatal POP exposure is related to altered fetal genital and reproductive tract development. However, the relationship between exposure to POP mixtures and anogenital distance (AGD) is poorly investigated. This study investigated the association between prenatal exposure to POP mixtures and AGD in 8-year-old children.

Methods: Data were collected from the INMA-Asturias cohort. Maternal serum POP concentrations were measured during the first trimester of pregnancy. Anoscrotal distance (AGD_{AS}) and anopenile distance (AGD_{AP}) in males and anofourchetal distance (AGD_{AF}) and anoclitoral distance (AGD_{AC}) in females were recorded in 362 8-years-olds. Conventional linear regression, and the novel weighted quantile sum regression (WQSR) and Bayesian kernel machine regression (BKMR) models were applied to assess the relationships between AGD and POPs exposure stratified by sex.

Results: Among males, in the linear regression, b-hexachlorocyclohexane, PCB138, PCB153, and PCB180 were inversely associated with the anogenital index $(AGI)_{AS}$ (−0.06 mm/kg (95% confidence interval [CI]: –0.11, −0.02), −0.07 mm/kg (95% CI: –0.14, −0.01), −0.07 mm/kg (95% CI: –0.13, −0.01), and −0.08 mm/kg (95% CI: –0.14, −0.02), respectively). Among females, polybrominated diphenyl ether (PBDE)47 and PBDE154 were positively associated with increased AGI_{AF} (0.02 mm/kg (95% CI: 0.00, 0.03) and 0.09 mm/kg (95% CI: 0.01, 0.17), respectively). BKMR confirmed these associations. WQSR found a negative combined effect of the POP mixture on AGD, and PCB138, PCB153, and PCB180 (weighted 0.18, 0.13, and 0.09, respectively) were identified as the most impacting chemicals. In females, WQSR found a positive combined effect and determined PBDE47 (weighted 0.35) as the most impacting.

Conclusions: Maternal exposure to a POP mixture was negatively associated with AGD in male children and positively associated with AGD in female children, thus providing evidence of the adverse effects of POPs on genital development.

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1. Introduction

Hexachlorobenzene (HCB), hexachlorocyclohexane (HCH), dichlorodiphenyltrichloroethane (44-DDT) and its primary metabolite dichlorodiphenyldichloroethane (44-DDE) constitute a group of organochlorine compounds (OCs). This group of compounds in addition to polychlorinated biphenyls (PCBs) are incorporated into the utero, ergo in the children's diet (Carrizo et al., 2006; Ribas-Fitó et al., 2006). Due to the lipophilic properties and high stability to chemical degradation of these compounds, they favor the accumulation of fat. Polybrominated diphenyl ethers (PBDEs), a class of brominated flame retardants, are used in plastics, textiles, furniture, and other manufacturing materials (Vuong et al., 2017); they are characterized by being highly persistent and slightly degradable in the environment and having a long biological life (2–12 years) and a remarkable bio-accumulative nature (Tiwari et al., 2018). Thanks to those properties that are characteristics of the group of persistent organic pollutants (POPs), it is possible to reckon PBDEs and other substances such as polyfluoroalkyl or dioxin and dioxin-like compounds in it.

OCs, PCBs and PBDEs can mimic or block the action of natural hormones (Heindel et al., 2015); thus, they can act as endocrine-disrupting chemicals (EDCs), altering critical biological processes of the endocrine system. The Stockholm Convention (2004) (SCOPS, 2004) banned their use and production; however, these compounds have persisted in the environment owing to their lipophilicity and ability to biomagnify (Casas et al., 2015). During prenatal exposure, POPs can be transmitted from mother to fetus through the placenta (Vizcaino et al., 2014a), and transmission continues postnatally through breastfeeding (Garí et al., 2019). Maternal and cord serum and placenta are the common matrices used to determine prenatal exposure to POPs (Barr et al., 2005). The main source of exposure in humans is through diet, particularly through contaminated food (Porta et al., 2008) or animal products, such as fish, meat, eggs, and dairy products (Costa et al., 2016; Llop et al., 2010).

The anogenital distance (AGD) is the distance between the genital tubercle and the center of the anus and is a sensitive marker of fetal androgen or anti-estrogenic action in children (Barrett et al., 2014; Dean and Sharpe, 2013). In mammals, the external genitalia and AGD length are sexually dimorphic, with the AGD being twice as long in males compared with females (Barrett et al., 2014; McIntyre et al., 2001). The *endocrine disruption hypothesis* (Vidaeff and Sever, 2005) postulates that environmental exposure to EDCs such as POPs alters a fetus's reproductive development by disrupting the androgen and estrogen receptors, leading to in utero hormonal imbalance. The masculinization programming window occurs during weeks 9–12 of pregnancy. This is a critical period during which POPs can act as anti-androgens, resulting in AGD alteration. Furthermore, AGD alteration is predictive of other androgen-responsive genitalia anomalies in male children such as hypospadias, cryptorchidism, and declining semen quality (Skakkebaek et al., 2001). A shorter AGD has been associated with these characteristics in male children (Drake et al., 2009; Singal et al., 2016; Thankamony et al., 2014). Similarly, in women, high testosterone levels and multifollicular ovaries are associated with a longer AGD (Mendiola et al., 2012; Mira-Escolano et al., 2014). Therefore, fetal POP exposure appears to affect genital development and can result in reproductive complications and other relevant health implications in adulthood.

Over the past 15 years, few epidemiological studies have evaluated the anti-androgenic effects of prenatal POP exposure on AGD, presumably because of the difficulty in obtaining reliable and contrasted measurements (García-Villarino et al., 2018, 2020; Lind et al., 2017; Longnecker et al., 2007; Loreto-Gómez et al., 2018; Luan et al., 2019, 2021; Torres-Sanchez et al., 2008). In general, the available literature has implicated the potential anti-androgenic effects of persistent organic congeners, with the most evidence for the adverse effects for PBDEs (Luan et al., 2019, 2021) and PCBs (García-Villarino et al., 2020). Other epidemiological studies have evaluated potential anti-androgenic effects of prenatal exposure to other EDC groups, with non-persistent organic pollutants, such as phthalates or Bisphenol-A (Barrett et al., 2017; Huang et al., 2009; Miao et al., 2011; Sun et al., 2018; Swan et al., 2005, 2015).

A major limitation is that research on how AGD is affected by prenatal POP exposure has focused on exposures to individual compounds. We currently lack information regarding the effects of exposure to POP mixtures during gestation, especially among populations exposed to relatively low concentrations. These effects can be evaluated by applying developed statistical approaches designed to assess nonlinear effects and interactions between mixture components. In real-world scenarios, people are exposed to mixtures of EDCs rather than to single compounds. Furthermore, it is difficult to discriminate the potential effects of exposure to individual compounds.

This study is among the first to assess the effects of prenatal exposure to POP mixtures and investigated whether the associations between prenatal POP exposure and AGD persist later in pre-puberty, an important period characterized by relevant changes in hormonal status. We explored the associations between prenatal exposure to a POP mixture (examining maternal serum concentrations of twelve organochlorine compounds [OCs] and six PBDE congeners) and AGD in children at 8 years of age.

2. Materials and methods

2.1. Study participants and design

Pregnant women visiting the San Agustin Hospital (Aviles) for their first prenatal care visit at 10–13 weeks of pregnancy were recruited from 2004 to 2007 and were enrolled as part of the INMA (INfancia y Medio Ambiente [Environment and Childhood]) mother–child cohort study (Fernández-Somoano et al., 2011; Riaño-Galán et al., 2017; Vizcaino et al., 2014a, 2014b). The eligibility criteria and details of participant withdrawal and loss to follow-up have been previously described (Fernández-Somoano et al., 2011; Fernández-Somoano and Tardon, 2014). Questionnaires on anthropometric and sociodemographic characteristics and lifestyle variables were performed in person with the assistance of trained personnel. 485 births took place between 2004 and 2008. Follow-up at 7–8 years was carried out to 416 children. Questionnaires on environmental health data including sociodemographic variables and diet were also performed. Three specialized paediatricians collected blood child samples and recorded anthropometric measures and genitalia measurements from each participant. AGD was measured in 332 children; of these, OCs were measured in 201 maternal serum samples (the OC dataset), and PBDEs were measured in 208 maternal serum samples (the PBDE dataset). Our complete dataset with no missing values in any POP exposure or covariates included 129 children (Figure S1). All participants signed the informed consent form prior to inclusion in the INMA project and then, after birth, signed a second consent form to include the newborns in the study. The research protocol and procedures were approved by The Ethical Committee of Regional Clinical Research.

2.2. Prenatal POP measurement

During the first trimester of gestation, 10–13 weeks, blood samples were collected by trained nurses following the INMA protocol (Guxens et al., 2012). Briefly, 5 mL of venous blood were collected and stored in the dark at 4 °C 1–2 h after collection (1–2 h from collection) until centrifugation for 15 min at 2500–3000 rpm. The serum obtained was aliquoted into 2-mL glass cryotubes and stored at −80 °C. The samples were transferred to the Barcelona Institute of Environmental Assessment and Water where they were posteriorly analyzed as previously described (Gari and Grimalt, 2010; Grimalt et al., 2010b; Vizcaino et al., 2009). The concentrations of twelve OCs were measured by gas chromatography with electron capture detection (Agilent Technologies 6890N gas

chromatograph; Santa Clara, CA, USA) using a DB-5 column protected by a retention gap (60 m × 0.25 mm id, 0.25-μm film thickness; J&W Scientific, Folsom, CA, USA). The OC limit of detection (LOD) was 0.010–0.035 ng/mL. Quantification was performed by the external standard method using these calibration lines and recovery (TBB and PCB-209) and injection (PCB-142) standards. The use of PCB-142 to correct for volume allows differentiating between corrections due to analyte losses by sample handling and volume variations in the final solvent rinsings for sample introduction into the chromatographic vials. More information about the quality control procedures have been previously described (Gari and Grimalt, 2010; Grimalt et al., 2010a). Serum concentrations of six PBDEs congeners were measured by gas chromatography coupled to mass spectrometry in chemical ionization mode using negative ion recording. The PBDE LODs ranged between 0.001 and 0.025 ng/mL. The General Biochemistry Laboratory of the San Agustín Hospital determined the concentrations of triglycerides and total cholesterol from the serum samples through colorimetric enzymatic methods. Methods described by Phillips and colleagues were used to determine total serum lipid concentrations (Phillips et al., 1989). The POP concentrations were normalized based on total serum lipids (total lipids = 2.27 × total cholesterol + triglycerides + 62.3 mg/dL) to account for bioaccumulation (Akins et al., 1989; Matta et al., 2022). Values of half the LOD were assigned when no measurable analytic concentration could be detected. Details of the protocols and instrumental analyses have been previously reported (García-Villarino et al., 2018, 2020).

2.3. AGD measurements in 8-year-old children

Child AGD assessment was performed according to the procedures described elsewhere (Salazar-Martinez et al., 2004; Swan et al., 2005, 2015) at the 7–8 year clinic visit. All measurements were performed by three examiners previously trained in the technique with Vernier calipers in increments of 0.1 mm. For female children, examiners measured the anofourchetal distance (AGD_{AF}), from the center of the anus to the posterior convergence of the fourchette, and the anoclitoral distance (AGD_{AC}), from the anterior surface of the clitoral hood to the center of the anus. For male children, the anopenile distance (AGD_{AP}) was measured from the center of the anus to the anterior base of the penis, and the anoscrotal distance (AGD_{AS}) was measured from the center of the anus to the junction of the smooth perineal skin with the rugated skin of the scrotum. Further details of the AGD measurements are in our previous study (García-Villarino et al., 2018, 2020; 2021a).

2.4. Covariates

Pregnant women completed two detailed questionnaires (at weeks 10–13 and 28–32) covering sociodemographic, environmental, and lifestyle factors. The questionnaires were administered in person by a trained interviewer. Maternal age at enrolment (years, continuous), maternal socioeconomic status (categorized as I–II [highest], III, or IV–V [lowest]), maternal education (categorized as primary, secondary, or university), gravidity (categorized as one, two, or three or more), maternal height (cm, continuous), pre-pregnancy body mass index (BMI) (categorized as underweight [<18.5 kg/m^2], normal [18.5–24.9 kg/m^2], overweight [25.0–29.9 kg/m^2], or obese [>30 kg/m^2]), smoking during pregnancy (yes vs. no), and passive smoke exposure during pregnancy (yes vs. no) were selected as potential confounders. Characteristics and demographic details of the children were gathered at the 7–9-year post-partum visit using questionnaires administered in person by a trained interviewer. The following variables were collected for the children: child age (years, continuous), sex (male *vs.* female, categorical), height (cm, continuous), and BMI (kg/m^2, continuous). Potential confounding variables were selected in accordance with prior studies and were based on their relationship with AGD as identified using the directed acyclic graph in the DAGitty browser-based environment (Textor et al., 2016).

2.5. Data analyses

Descriptive statistics were calculated for the continuous variables. Counts and percentages [*n* (%)] were used for the categorical variables. Prenatal POP serum concentrations were natural-log transformed to reduce their skewness. The POP concentrations were also centered and scaled, and Spearman's coefficients were calculated for each compound pair. We calculated the anogenital index (AGI) described by Swan and colleagues (Swan et al., 2005), AGI = AGD/weight at 8 years (mm/kg). The POP concentrations were adjusted by total serum lipid concentrations (ng/g lipid) and used in the data analyses.

Covariate-adjusted linear regression analyses were performed to quantify the effects of individually prenatal POP exposure on the AGIs. The linear regression models used the POP concentrations, normalized by their interquartile range (IQR) (i.e., POP/IQR[POP]), as independent variables. The AGIs were used as the dependent variables and were adjusted for maternal age, gravidity, smoking during pregnancy, passive smoke exposure during pregnancy, maternal height, pre-pregnancy BMI, child's height at 8 years, and examiner (Figure S2).

As a complement, to evaluate the effect of the POP mixture on AGD, we implemented weighted quantile sum regression (WQSR) (Carrico et al., 2015) and Bayesian kernel machine regression (BKMR) (Bobb et al., 2014). The WQSR models were used to evaluate the impact of the POP mixture and to identify the most impacting compounds. In the current analysis, compounds with estimated weights greater than 0.10 were considered to significantly contribute to the WQSR score. The WQSR approach assumed that all mixture components acted in the same directionality on AGD. Positive and negative WQSR scores were calculated for females and males, respectively. Weights for the WQSR scores were computed using 100 bootstrap samples. We performed adjusted BKMR modelling to evaluate the joint effect of the POP mixture. This approach allowed us to determine possible interactions and nonlinear associations. All BKMR models used a Gaussian kernel with 5000 as the burn-in and 50,000 Monte Carlo iterations with Markov chain. Additional details on the BKMR procedure are previously described (García-Villarino et al., 2021b; Signes-Pastor et al., 2020). Both statistical approaches were adjusted for the covariates described above.

R statistical software version 3.5.1 (R Core Team, 2014) was used for the data analyses. Specifically, the *bkmr* (Bobb et al., 2014) and *gWQS* (Carrico et al., 2015) R packages were used.

3. Results

Demographic characteristics of the participants are presented in Table 1. The median maternal age and height were 31 years and 163 cm, respectively. Overall, 26% of the pregnant women were overweight or obese, 25.6% smoked during pregnancy, and 42.6% had passive smoke exposure during pregnancy. No disorders or genital malformations were observed in the children enrolled in this study. In female children, the median AGD_{AF} and AGD_{AC} were 20 and 92 mm, respectively. The median AGI_{AF} and AGI_{AC} were 0.7 mm/kg and 3.0 mm/kg, respectively. In male children, the median AGD_{AS} and AGD_{AP} were 34.0 and 110.0 mm, respectively. The median AGI_{AS} and AGI_{AP} were 1.2 mm/kg and 3.7 mm/kg, respectively (Figure S2). The descriptive analysis results were similar among the OC and PBDE datasets (Table 1). Children excluded from the study do not differ in demographic and anthropometric variables from those children who have been included in the main analysis ($n = 82$, Table S1).

The maternal serum concentrations of chemicals are summarized in Table 2. HCB; b-HCH; 44-DDE, 44-DDT, PCB118, PCB138, PCB153, PCB180, PBDE153, and PBDE154 were detected in over 85% of study participants. The detection rates of the other analyzed congeners ranged from 15% to 73%. The heat map of Spearman's correlation coefficients showed weakly negative to strongly positive correlations. In particular,

Table 1
Selected characteristics of the INMA-Asturias study population.

	OCs dataset (n = 201)	PBDEs dataset (n = 208)	Complete dataset (n = 129)
Children characteristics			
Male/Female	99 (49.0%)/102 (51.0%)	111 (53.0%)/97 (47.0%)	64 (49.0%)/65 (51.0%)
Age (years)	8.0 (7.0, 8.0–8.0, 9.0)	8.0 (7.0, 8.0–8.0, 9.0)	8.0 (7.0, 8.0–8.0, 9.0)
Height (cm)	131.1 (113.0, 126.9–134.6, 149.8)	130.7 (113.0, 126.6–134.2, 149.8)	131.3 (113.0, 127.0–134.5, 149.8)
Weight (cm)	29.2 (19.4, 26.1–34.4, 52.1)	29.1 (19.4, 25.9–33.5, 54.0)	29.7 (19.4, 26.0–33.9, 51.0)
BMI (kg/m^2)	17.3 (13.1, 15.7–19.4, 28.1)	17.1 (12.8, 15.6–19.2, 28.6)	17.4 (13.1, 15.7–19.3, 27.4)
AGD at 8 years (mm)			
AGD_{AS} (male)	34.0 (16.0, 27.5–42.0, 65.0)	33.0 (14.0, 26.5–40.0, 65.0)	34.0 (16.0, 27.0–41.2, 65.0)
AGD_{AP} (male)	110.0 (58.0, 99.0–120.5, 145.0)	110.0 (58.0, 99.5–118.0, 139.0)	110.0 (58.0, 98.0–120.0, 139.0)
AGD_{AF} (female)	20.0 (9.0, 16.0–24.0, 55.0)	19.0 (9.0, 16.0–22.0, 47.0)	20.0 (9.0, 16.0–24.0, 42.0)
AGD_{AC} (female)	93.5 (46.0, 83.0–104.0, 130.0)	91.0 (46.0, 80.0–100.1, 130.0)	92.0 (46.0, 80.0–104.0, 130.0)
AGI at 8 years (mm/kg)			
AGI_{AS} (male)	1.1 (0.5, 0.9–1.4, 2.0)	1.1 (0.4, 0.9–1.4, 2.1)	1.2 (0.5, 0.9–1.4, 2.0)
AGI_{AP} (male)	3.7 (1.8, 3.2–4.1, 4.9)	3.8 (1.9, 3.3–4.1, 5.4)	3.7 (1.9, 3.2–4.0, 4.9)
AGI_{AF} (female)	0.7 (0.3, 0.6–0.8, 2.1)	0.7 (0.2, 0.6–0.8, 1.3)	0.7 (0.3, 0.6–0.8, 1.1)
AGI_{AC} (female)	3.1 (1.6, 2.8–3.5, 4.3)	3.0 (1.8, 2.8–3.4, 4.4)	3.0 (1.8, 2.8–3.4, 4.1)
Maternal characteristics			
Age (years)	31.0 (19.0, 29.0–35.0, 42.0)	32.0 (19.0, 29.0–35.0, 42.0)	31.0 (19.0, 29.0–35.0, 42.0)
Height (cm)	163.0 (147.0, 160.0–167.0, 184.0)	162.0 (150.0, 159.8–166.0, 184.0)	163.0 (150.0, 160.0–167.0, 184.0)
Gravidity			
One	104 (52.0%)	105 (50.4%)	66 (51.2%)
Two	56 (28.0%)	64 (30.8%)	33 (25.6%)
Three or more	41 (20.0%)	39 (18.8%)	30 (23.2%)
Pre-pregnancy BMI			
Underweight (<18.5 kg/m^2)	7 (3.0%)	8 (3.8%)	3 (2.3%)
Normal (18.5–24.9 kg/m^2)	135 (67.0%)	144 (69.2%)	92 (71.3%)
Overweight (25.0–29.9 kg/m^2)	41 (21.0%)	44 (21.2%)	26 (20.2%)
Obese (≥30 kg/m^2)	18 (9.0%)	12 (5.8%)	8 (6.2%)
Maternal social class			
I-II (highest)	33 (16.5%)	49 (23.6%)	24 (18.6%)
III	54 (26.9%)	49 (23.6%)	35 (27.1%)
IV-V (lowest)	110 (54.7%)	109 (52.4%)	69 (53.4%)
Maternal education			
Primary	28 (14.0%)	26 (12.5%)	18 (13.9%)
Secondary	91 (45.0%)	92 (44.2%)	57 (44.2%)
University	82 (41.0%)	90 (43.3%)	54 (41.9%)
Smoke during pregnancy			
Yes	50 (24.9%)	55 (26.4%)	33 (25.6%)
No	145 (72.1%)	150 (72.1%)	94 (72.9%)
Maternal passive smoking			
Yes	90 (44.8%)	93 (44.7%)	55 (42.6%)
No	105 (52.3%)	112 (53.8%)	71 (55.1%)

Categorical variables = n (%); Continuous variables = median (min., percentile 25 – percentile 75, max.); BMI = body mass index. AGD = anogenital distance; AGI = anogenital index; OCs = organochlorine compounds; PCB = polychlorinated biphenyl; PBDE = polybrominated diphenyl ethers. AGI_{AS} = Anoscrotal index; AGI_{AP} = Anopenile index. AGI_{AF} = Anofourchetal index; AGI_{AC} = Anoclitoral index.

PCB153 was strongly positively correlated with PCB138 ($\rho > 0.9$). b-HCH and HCB were also positively correlated ($\rho > 0.7$), and PBDE153 and PBDE154 were positively correlated ($\rho > 0.6$). The correlation coefficients ranged from −0.32 to 0.96 (Figure S3).

Conventional linear regression methods were used to quantify the associations between prenatal POP exposure and AGI. We evaluated OCs/PCBs and PBDEs individually and adjusted for the previously described covariates. Fig. 1A shows the associations between prenatal OCs and PBDEs (OCs and PBDEs dataset, respectively) exposure and the AGIs for male children at 8 years. An IQR increase in the maternal serum concentrations of b-HCH, PCB138, PCB153, and PCB180 was associated with a −0.06 mm/kg (95% CI: −0.11, −0.02), −0.07 mm/kg (95% CI: −0.14, −0.01), −0.07 mm/kg (95% CI: −0.13, −0.01), and −0.08 mm/kg (95% CI: −0.14, −0.02) decrease in AGI_{AS}, respectively. An inverse association was also found for every IQR increment in PBDE99 concentration, the average AGI_{AS} for the complete sample dataset decreased by −0.15 mm/kg (95% CI: −0.29, −0.01) (Fig. 1B). Among females, for every IQR increase in maternal serum PBDE47 and PBDE154 concentration (PBDEs dataset), there was a 0.02 mm/kg (95% CI: 0.00, 0.03) and 0.09 mm/kg (95% CI: 0.01, 0.17) increase in AGI_{AF}, respectively (Fig. 2A). Furthermore, PBDE209 was positively associated with an increased AGI_{AC} of 0.09 mm/kg (95% CI: 0.01, 0.18). In the complete sample dataset, we found a positive association between 44-DDT and AGI_{AC}: for every IQR increment in 44-DDT concentration, the average AGI_{AC} increased by 0.15 mm/kg (95% CI: 0.04, 0.26) (Fig. 2B).

Fig. 3 shows the estimated compound weight for each WQSR score. In male children, b-HCH had the highest weight related to AGI_{AS} (weighted 0.26), followed by PCB118, PCB180, and PCB153 (weighted 0.18, 0.13, and 0.09, respectively). PBDE-99 was the most important congener among the PBDEs (weighted 0.43). For the AGI_{AP}, 44-DDE and PBDE99 had the highest weights (weighted 0.24 and 0.49, respectively). In female children, for the AGI_{AF} subset, 44-DDT and PBDE47 had the highest weights (weighted 0.24 and 0.35, respectively). b-HCH and PBDE28 had the highest weights in the AGI_{AC} model (weighted 0.17 and 0.45, respectively). The complete dataset shows that PCB118 and PBDE99 had the highest weight related to AGI_{AS} and AGI_{AP}, respectively for male children (weighted 0.21 and 0.27). Regarding female children, PCB28 and PBDE209 had the highest weight related to AGI_{AS} and AGI_{AP}, respectively (weighted 0.16 and 0.21).

Fig. 4 displays the BKMR univariate exposure–response functions among male children. It shows the associations between each POP, with the other pollutants fixed at the median, and AGI_{AS} (Fig. 4A) and AGD_{AP} (Fig. 4B). Generally, the associations between each POP exposure and AGI_{AS} and AGD_{AP} appeared to be linear when considering the POP mixture effect. There was some evidence of nonlinear effect of b-HCH and PCB180 with AGI_{AS} but with wide confidence bands especially at low concentrations. Among female children, the univariate exposure–response functions (Fig. 5) also had an overall linear trend with AGI_{AF} (Fig. 5A) and AGD_{AC} (Fig. 5B). The association between PCB180 with the other POPs fixed at the median and AGI_{AF} showed evidence of nonlinear effect but with high variability in line with the male children's findings. The BKMR analyses did not show evidence of interaction between POPs congeners (data not shown). The results from the complete dataset (Figure S5 and Figure S6) shows that the associations between each POP exposure and AGI_{AS} and AGD_{AP} appeared to be linear when considering the POP mixture effect for both, males and females. The overall POPs mixture exposure effect is reported in supplemental information (Figure S7 and Figure S8). Figure S7 and Figure S8 plot the estimated change in AGI index for males and female's children, respectively, when POP exposures are at a particular percentile as shown in the x-axis compared to when exposure are all at the 50th percentile. The joint mixture effect suggests an increased toxicity trend; however, it did not reach statistical significance for either male or female infants.

Table 2
Detection rate and POP concentrations (ng/g lipid) in maternal blood serum.

	n	LOD (ng/ml)	% (>LOD)	Min	5th	25th	Median	75th	95th	Max
HCB	201	0.0151	100	23.89	3.95	45.69	69.82	110.71	210.32	945.37
b-HCH	201	0.001	99.01	4.98	0.12	17.26	27.23	46.82	86.87	322.81
g-HCH	201	0.0183	15.42	1.73	1.12	2.20	2.49	2.95	8.43	24.12
44-DDD	201	0.003	34.32	0.23	0.14	0.29	0.35	1.04	3.88	26.92
44-DDE	201	0.0081	99.00	59.82	0.55	169.33	280.78	490.41	1441.52	3945.32
44-DDT	201	0.0247	100	3.99	1.55	10.67	18.78	29.64	85.33	388.04
PCB28	201	0.0064	72.63	0.40	0.20	0.57	3.76	7.71	20.25	43.45
PCB101	201	0.0225	56.71	1.14	0.73	1.53	5.11	12.42	25.00	55.94
PCB118	201	0.0286	93.53	1.54	0.97	5.98	10.07	16.99	27.42	47.72
PCB138	201	0.0173	100	14.20	1.60	26.72	36.54	56.62	80.77	163.98
PCB153	201	0.0215	100	29.39	8.59	49.54	62.54	89.89	121.38	253.44
PCB180	201	0.0103	100	12.18	1.79	32.36	45.96	66.57	108.17	275.96
PBDE28	208	0.0015	23.56	0.12	0.07	0.15	0.15	0.20	1.43	2.61
PBDE47	208	0.0025	19.71	0.21	0.12	0.25	0.25	0.31	5.77	24.83
PBDE99	208	0.0023	59.62	0.21	0.11	0.23	1.50	3.70	10.30	30.59
PBDE153	208	0.0027	95.19	0.42	0.10	1.74	2.62	4.74	9.68	84.29
PBDE154	208	0.0012	84.61	0.27	0.14	1.21	2.59	5.57	11.22	27.32
PBDE209	208	0.0091	31.73	0.75	0.47	0.89	0.89	2.74	14.56	38.95

HCB = hexachlorobenzene; HCH = hexachlorocyclohexane; 44-DDD = 1,1-bis(4-chlorophenyl)-2,2-dichloroethane; 44-DDE = 2,2-bis(4-chlorophenyl)-1,1-dichloroethene; 44-DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; PCB, polychlorinated biphenyl; PBDE, polybrominated diphenyl ethers. LOD = limit of detection; 5th = percentile 5; 25th = percentile 25; 75th = percentile 75-; 95th = percentile 95.

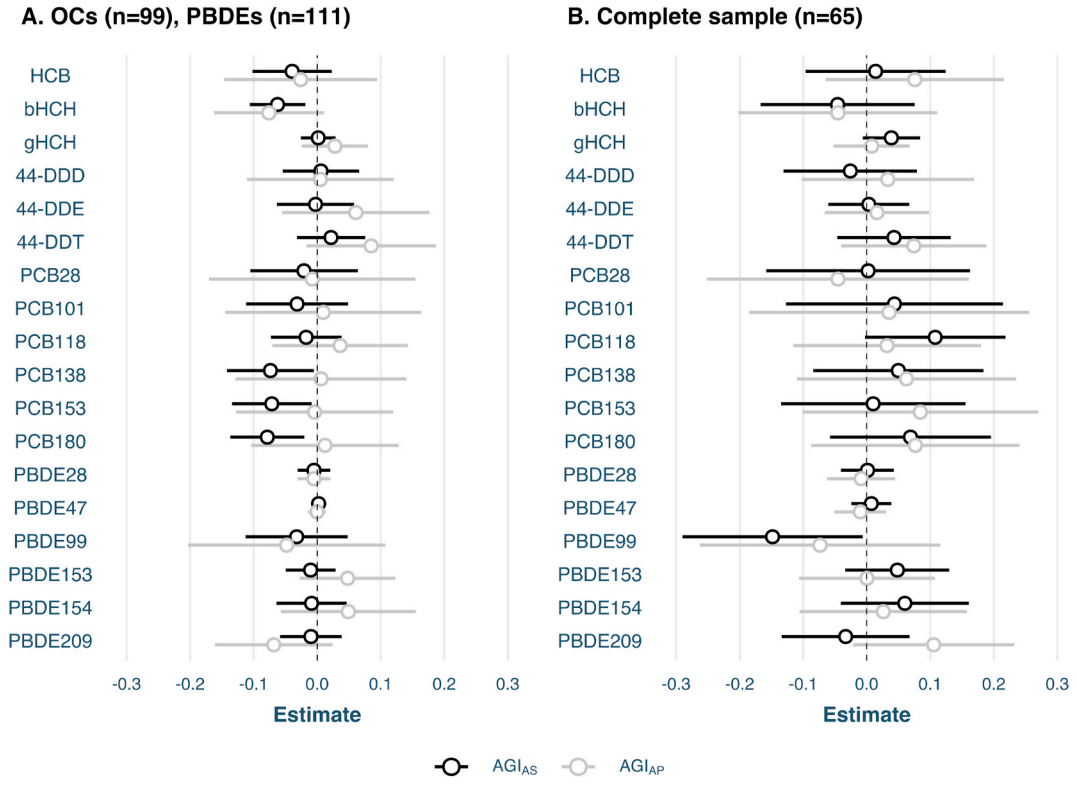

Fig. 1. Changes in male children's AGI_{AS} and AGI_{AP} for each IQR increase in maternal serum concentrations of organochlorine compounds and polybrominated diphenyl ethers. HCB = hexachlorobenzene; HCH = hexachlorocyclohexane; 44-DDD = 1,1-bis(4-chlorophenyl)-2,2-dichloroethane; 44-DDE = 2,2-bis(4-chlorophenyl)-1,1-dichloroethene; 44-DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; PCB, polychlorinated biphenyl; PBDE, polybrominated diphenyl ethers. AGI_{AS} = Anoscrotal index; AGI_{AP} = Anopenile index. Models adjusted for maternal age, gravidity, smoking during pregnancy, passive smoke exposure during pregnancy, maternal height, pre-pregnancy BMI, children height at 8-years and examiners.

4. Discussion

This study investigated the potential effects of prenatal exposure to a POP mixture on the AGIs of 8-year-old children enrolled in the INMA-Asturias cohort using both traditional and newer statistical approaches. Conventional linear regression, WQSR, and BKMR were used to evaluate the associations between the concentrations of 18 POPs and the AGIs. Among male children, negative linear dose–response associations were found in the linear regression analyses. WQSR and BKMR confirmed the negative combined effect of POP mixture exposure on the AGIs. In female children, all approaches found positive associations between some compounds and the AGIs, and BKMR showed a general linear dose–response relationship between POPs and the AGIs. WQSR also confirmed the positive combined effect of the multiple co-exposures. BKMR was used to estimate a flexible exposure-response surface, potential interactions, single-exposure effect, and overall

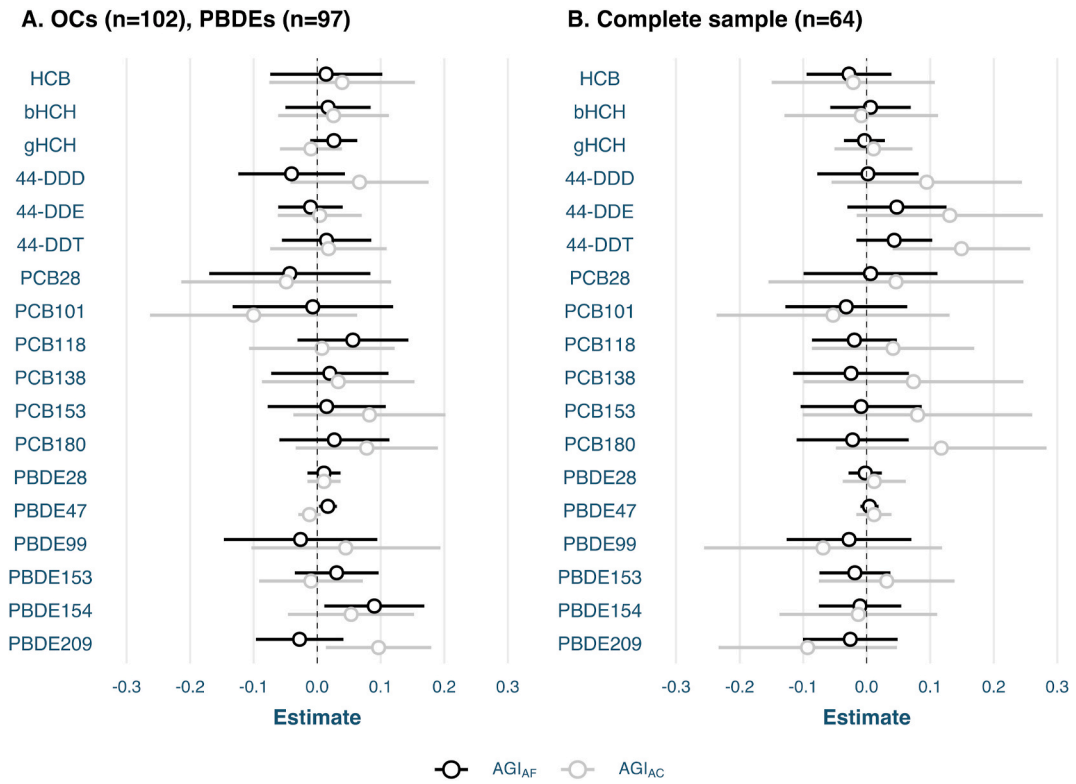

Fig. 2. Changes in female children's AGI_{AS} and AGI_{AP} for each IQR increase in maternal serum concentration of organochlorine compounds and polybrominated diphenyl ethers. HCB = hexachlorobenzene; HCH = hexachlorocyclohexane; 44-DDD = 1,1-bis(4-chlorophenyl)-2,2-dichloroethane; 44-DDE = 2,2-bis(4-chlorophenyl)-1,1-dichloroethene; 44-DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; PCB, polychlorinated biphenyl; PBDE, polybrominated diphenyl ethers. AGI_{AF} = Anofourchetal index; AGI_{AC} = Anoclitoral index. Models adjusted for maternal age, gravidity, smoking during pregnancy, passive smoke exposure during pregnancy, maternal height, pre-pregnancy BMI, children height at 8-years and examiners.

mixture effect. Moreover, WQSR can quantify the joint effect of the mixture and identify the important contributors to the effect. These approaches allow for testing both the overall mixture effect and the effects of each mixture component within the context of the overall joint exposure.

The linear regression analyses showed a negative association between the AGI_{AS} in male children and prenatal concentrations of PCB138, PCB153, PCB180, and b-HCH. We also found that PBDE99 was negatively associated with AGI_{AP}. These findings are in line with our previous studies conducted in children at 18 months (García-Villarino et al., 2018) and 4 years of age (García-Villarino et al., 2020). The trends suggest that POPs affect fetal development through an androgen action during the masculinization programming window. The present findings are also in line with those of previous studies (Loreto-Gómez et al., 2018; Luan et al., 2019). Loreto-Gomez and colleagues reported negative associations between AGD/height at 12 months of age and maternal serum concentrations of PCB28, PCB74, and PCB-70 collected during the third trimester of pregnancy (Loreto-Gómez et al., 2018). A study from China also reported negative associations between AGD_{AS} at 12 and 48 months and cord plasma concentrations of PBDE-47 (Luan et al., 2019).

Considering that POP co-exposures might interact to produce confounding or additive effects, we used WQSR and BKMR to investigate the joint effects of the POP mixture. This was done to account for the limitations of the interactions and the linearity of conventional analysis because in the real world, pregnant women are exposed to a mixture of EDCs during pregnancy. In this analysis, PCB138, PCB153, and PCB180 were also found to be the congeners in the mixture that had the strongest effect on decreasing the AGIs in male children at 8-year-olds. To date, the literature has focused on individual prenatal POP exposures and their association with AGD in male children (Bornman et al., 2016; Lind et al., 2017; Longnecker et al., 2007; Loreto-Gómez et al., 2018; Luan et al., 2019, 2021; Torres-Sanchez et al., 2008). Therefore, the mixed effects found in the current study should be interpreted with caution. Furthermore, when we used all 18 compounds in the same model, our sample size was considerably reduced, which limited our ability to identify the smaller impacts that would likely become apparent in a larger sample size. Further studies that focus on multiple co-exposures are needed to validate our findings.

At present, the mechanisms by which POPs might play a role in genital development are not well characterized. One possible mechanism is that POPs might downregulate the proteins responsible for steroidogenesis, or they might hinder the action of androgen (Lardone et al., 2011; Stickels et al., 2015). Another proposed biological pathway is that POPs might modulate the production of testosterone in Leydig cells (Zhang et al., 2017). Studies have shown that decreased prenatal androgen concentrations are associated with decreased AGD (Moore et al., 2001). Anti-androgenic properties have been well described for POPs in *in vitro* and *in vivo* assays (Dang et al., 2007; Hamers et al., 2006; Meerts et al., 2001). Therefore, a plausible biological explanation is that POPs (i.e., PCBs, DDT, or PBDEs) act through an anti-androgenic effect. However, PBDEs impact the estrogen receptor through agonistics responses (Hamers et al., 2006; Liu et al., 2011). Sathyanarayana and colleagues found that estrogen signaling during genital development might be regulated by a minor allele located in the coding region of the estrogen receptor and that estrogen signaling was associated with a shorter AGD (Sathyanarayana et al., 2012).

Among female children, conventional analyses have found consistently positive associations between AGI_{AF} and the congeners PBDE47 and PBDE154. Moreover, PBDE209 and 44-DDT have also been found to be positively associated with AGI_{AC}. In addition, PBDE47 was previously shown to be the predominant element in a mixture of PBDEs that increased AGI_{AF}. A study from China reported a positive relationship

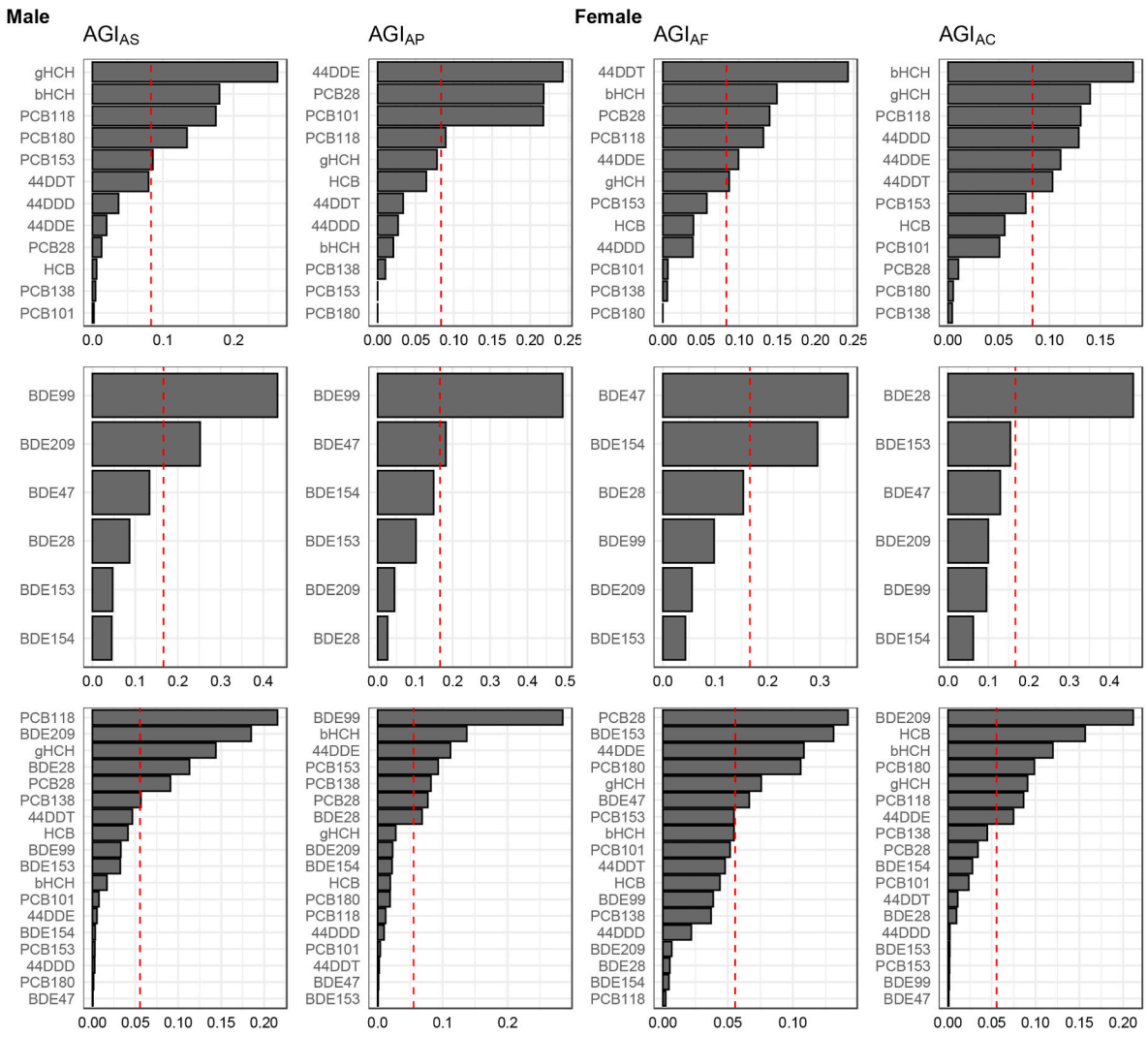

Fig. 3. Associations between POPs and AGIs in 8-year-old children by WQS regression. HCB = hexachlorobenzene; HCH = hexachlorocyclohexane; 44-DDD = 1,1-bis (4-chlorophenyl)-2,2-dichloroethane; 44-DDE = 2,2-bis(4-chlorophenyl)-1,1-dichloroethene; 44-DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; PCB, polychlorinated biphenyl; PBDE, polybrominated diphenyl ethers. Positive WQS scores were calculated for females and negative WQS scores were performed for male children. AGI_{AS} = Anoscrotum index; AGI_{AP} = Anopenile index. AGI_{AF} = Anofourchette index; AGI_{AC} = Anoclitoral index. Models adjusted for maternal age, gravidity, smoking during pregnancy, passive smoke exposure during pregnancy, maternal height, pre-pregnancy BMI, children height at 8-years and examiners. Notice that the scale of the x- and y-axis vary in order to facilitate the visualization of the estimates in each plot.

between increased AGD_{AF} at ages 0–4 years and prenatal exposure to PBDE47 (Luan et al., 2021). That study also reported a similar association between PBDE153 and AGD_{AC}. These findings were consistent with our PBDE154 results and for the analyses of the PBDE mixture dataset. Regarding 44-DDT and its derivatives, increased 44-DDT concentrations were associated with increased AGI_{AC}. This is supported by our prior study where an increased AGI_{AF} at 4 years of age was associated with prenatal 44-DDT concentration (García-Villarino et al., 2020). In general, we observed positive associations between the AGIs and prenatal exposure to POPs. These associations are among the earliest described in the literature to date, and thus cannot be robustly compared with other studies. However, the mixture analysis conducted here was in line with our previous findings and enabled us to determine the most important compounds, which we subsequently quantified in a conventional analysis.

The biological mechanisms that explain the associations between prenatal exposure to POPs and AGD in female children are not as clear as they are for male children. The anti-androgenic and estrogenic properties of POPs, including PBDES, have been thoroughly examined in *in vivo* and *in vitro* studies (Dang et al., 2007; Meerts et al., 2001). The literature also shows that estrogenic action seems to be necessary for proper sexual development in females, whereas in males, anti-androgenic properties are more related to genital development (Yang et al., 2010). Although fetal exposure to ethinylestradiol does not affect male AGD in rats, female offspring may exhibit longer AGD after exposure to supra-physiological doses (Casanova et al., 1999; Delclos et al., 2009; Mandrup et al., 2013; Ryan et al., 2010), similarly in humans, prenatal exposure to estrogenic chemicals (such as 44-DDT) has been associated with increased AGD in female children (García-Villarino et al., 2020). Moreover, Barrett and colleagues hypothesized that AGD is altered by prenatal exposure to EDCs through estrogenic mechanisms, which is line with our findings (Barrett et al., 2017). This effect seems counterintuitive; however, very high doses of steroidal estrogens can agonize the androgen receptor (Vinggaard et al., 1999), suggesting that the effects are driven by androgen action rather than being estrogenic effects. This mechanism would support our findings and those of Luan and colleagues (Luan et al., 2021), where exposure to POPs during pregnancy was associated with increased AGD. *In vivo* and *in vitro* studies have shown that PBDE153 can inhibit estradiol-induced activity, showing an anti-estrogenic effect (Meerts et al., 2001; Navas and Segner, 1998).

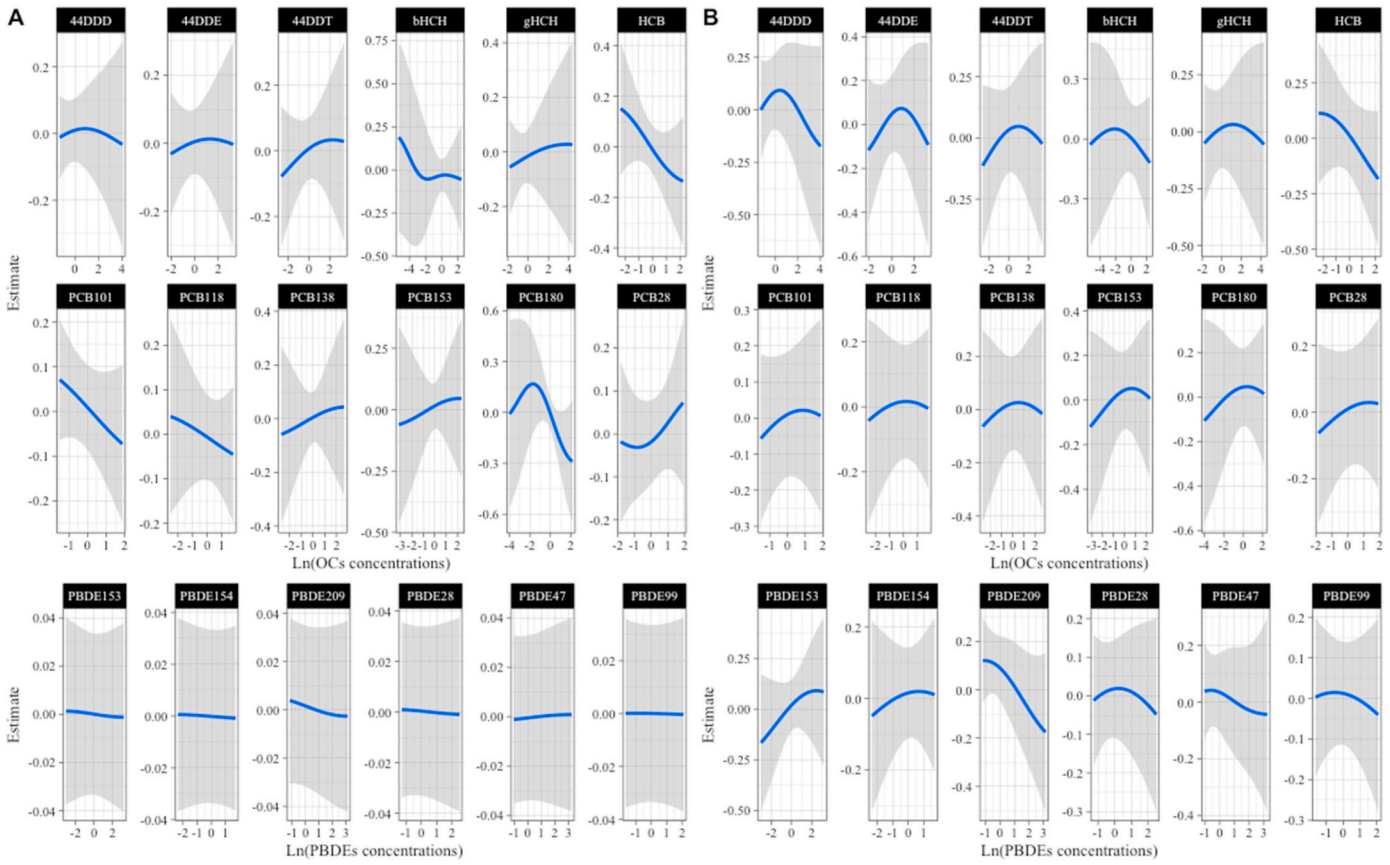

Fig. 4. BKMR univariate exposure–response functions and 95% confidence intervals for each POP, with the other congeners fixed at the median for male children (OCs dataset n = 99; PBDEs dataset n = 111). **A**, Univariate exposure-response functions and 95% confidence bands for each compound with the other pollutants fixed at the median for the AGI_{AS} index. **B**, Univariate exposure-response functions and 95% confidence bands for each compound with the other pollutants fixed at the median for the AGI_{AP} index. HCB = hexachlorobenzene; HCH = hexachlorocyclohexane; 44-DDD = 1,1-bis(4-chlorophenyl)-2,2-dichloroethane; 44-DDE = 2,2-bis(4-chlorophenyl)-1,1-dichloroethene; 44-DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; PCB, polychlorinated biphenyl; PBDE, polybrominated diphenyl ethers. Models adjusted for maternal age, gravidity, smoking during pregnancy, passive smoke exposure during pregnancy, maternal height, pre-pregnancy BMI, children height at 8-years and examiners. Notice that the scale of the x- and y-axis vary in order to facilitate the visualization of the estimates in each plot.

However, anti-estrogenic actions might affect the processes of both growth and sexual development.

The present study had some limitations. First, our sample size of 129 mother–child pairs may not be sufficient and provides limited statistical power for examining differences among the mixture of POPs and their individual impacts on AGD. Second, the serum POP concentrations found in this study were lower than in previously examined populations (Loreto-Gómez et al., 2018; Luan et al., 2019), which may limit our capacity to quantify associations that would occur at higher concentrations. The differences in POP concentrations can be attributed to the different times of sampling (first *vs*. third trimester) or the different matrices tested (maternal serum *vs*. cord serum). Third, three specialized paediatricians assessed the two AGD metrics, leading to possible variability in the results. However, inter-examiner reliability was tested for 10% of the measurements, and no significant variability was detected. Fourth, the placenta plays a crucial role during fetal development and has the capability to the production of a wide variety of hormones (including androgens and estrogens) and other regulatory factors, and is also an endocrine target tissue, expressing hormone receptors and growth factor receptors (Sathishkumar et al., 2012). Thus, a complex interplay of hormones and other regulatory factors produced by the placenta, mother and fetus affect placental development and functions through endocrine mechanisms. Normally, androgens synthesized by the placenta are rapidly converted to estrogens by placental aromatase, and therefore the placental androgens may contribute slightly to the increased androgen observed in normal pregnancy (Buster et al., 1979). Furthermore, there is evidence that POPs have the capacity to transfer to the fetus through the placenta (Vizcaino et al., 2014a). However, it is not known whether POPs could affect the production of hormones by the placenta, which may subsequently affect AGD of fetus. One last limitation that we have found, as we have gone deeper into the study of AGD throughout the different visits to the paediatrician (18 months, 4 and 8 years), is that we have observed a reduction in the number of participants who were willing to measure AGD. This fact is marked by child growth and development. The present work reflects an age in which children begin to know their own bodies (prepubertal state) and, on occasions, become more reluctant to have their intimate areas explored. If we add to these facts the psychosocial factor that the results obtained from the measurements may be adverse and may be related to reproductive health, it is more difficult for the degree of satisfaction on the part of the participants to be maintained over time.

This study also had several strengths. First, to our knowledge, the INMA-Asturias cohort is the first to identify the effects of multi-pollutant exposures on AGD, thus providing an additional reference for evaluating the health effects of exposure to multiple POPs. Second, our research was based on a well-designed cohort study with maternal samples collected from early pregnancy, which is one of the most relevant periods for fetal reproductive system development. Third, this study obtained information on AGD in 8-year-old children, an age at which this has rarely been studied.

AGD has been used as a non-invasive biomarker in recent years and due to its clinical relevance is likely to continue to be used in the future.

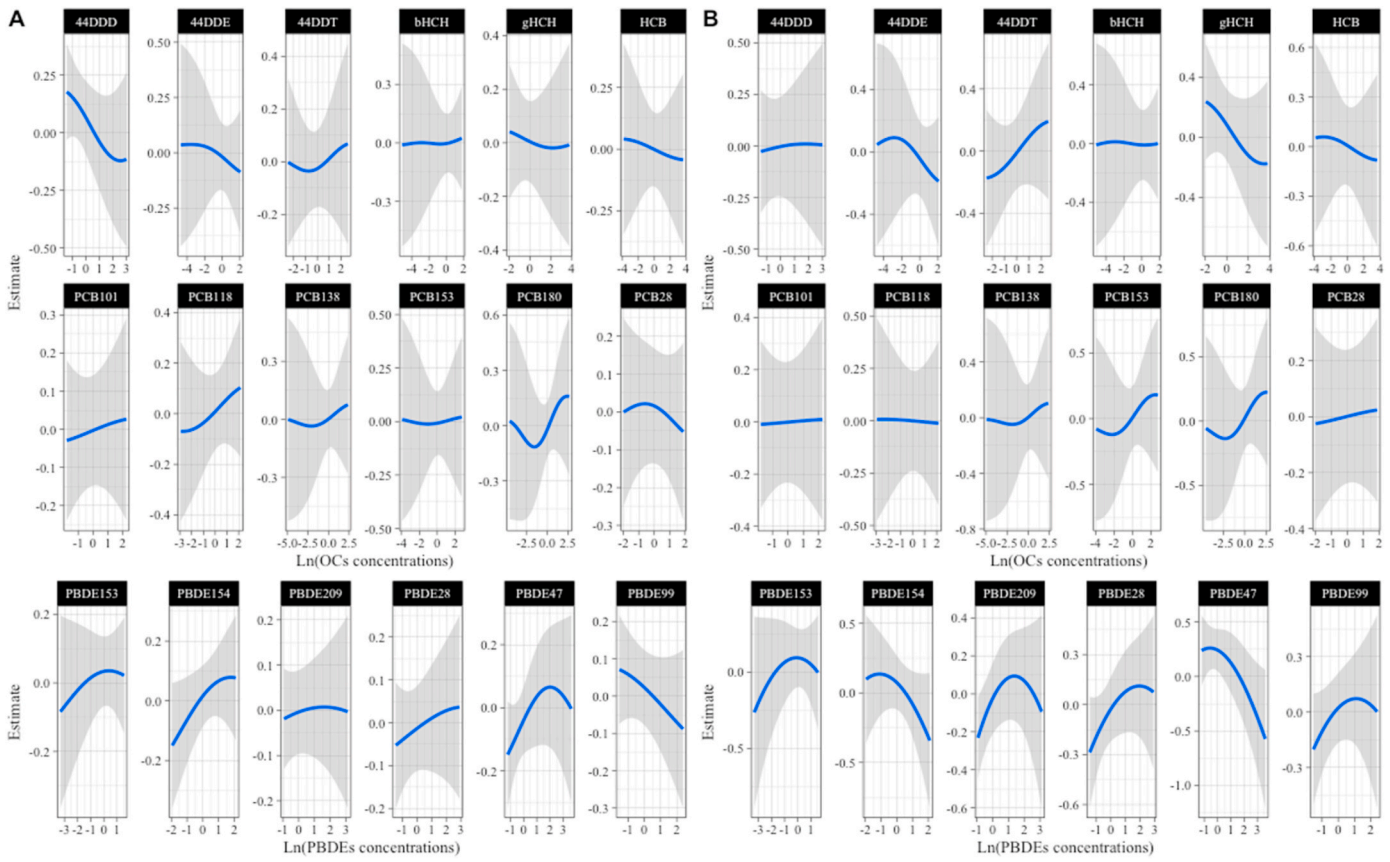

Fig. 5. BKMR univariate exposure-response functions and 95% confidence intervals for each POP, with the other congeners fixed at the median for female children (OCs dataset n = 102; PBDEs dataset n = 97). **A**, Univariate exposure-response functions and 95% confidence bands for each compound with the other pollutants fixed at the median for the AGI_{AF} index. **B**, Univariate exposure-response functions and 95% confidence bands for each compound with the other pollutants fixed at the median for the AGI_{AC} index. HCB = hexachlorobenzene; HCH = hexachlorocyclohexane; 4,4′-DDD = 1,1-bis(4-chlorophenyl)-2,2-dichloroethane; 4,4′-DDE = 2,2-bis(4-chlorophenyl)-1,1-dichloroethene; 4,4′-DDT = 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane; PCB, polychlorinated biphenyl; PBDE, polybrominated diphenyl ethers. Models adjusted for maternal age, gravidity, smoking during pregnancy, passive smoke exposure during pregnancy, maternal height, pre-pregnancy BMI, children height at 8-years and examiners. Notice that the scale of the x- and y-axis vary in order to facilitate the visualization of the estimates in each plot.

However, the purposes for which it will be used may change. It is now considered a robust biomarker for reproductive toxicity studies, particularly in animals, in that a short male AGD in neonates indicates insufficient androgen signaling during critical developmental stages, whereas a long female AGD indicates excess androgen signaling. Furthermore, there is a clear association with other reproductive disorders in both sexes in both rodents and humans, making AGD of eloquent value in chemical evaluations for potential endocrine-disrupting activities. Therefore, it is important to identify which prenatal exposures may have the greatest impact on the AGD of offspring and to study the problem from a multiple exposure perspective. This field is yet to be explored; thus, further studies are needed to verify the identified associations between prenatal exposure and AGD.

In conclusion, the conventional linear regression, WQSR, and BKMR models found that co-exposure to the investigated compounds was associated with reduced AGIs in male children. In contrast, the combined exposure to POPs was associated with increased AGIs in female children. These co-exposures might affect genital development and lead to reproductive complications, with important health implications in adulthood. Further studies are needed to verify the identified associations between prenatal exposure and AGD.

Author contributions

Miguel García-Villarino: formal analysis, investigation, data curation, methodology, visualization, writing-original draft, writing–review & editing. **Antonio J. Signes-Pastor**: conceptualization, investigation, methodology, supervision, writing–review & editing. **Isolina Riaño-Galán**: data collection, writing–review & editing. **Cristina Rodríguez-Dheli**: data collection. **Esther Vizcaíno**: methodology, resources. **Joan O. Grimalt**: methodology, resources, writing–review & editing. **Ana Fernández-Somoano**: conceptualization, investigation, methodology, supervision, writing–review & editing. **Adonina Tardón**: supervision, project administration, funding acquisition, writing–review & editing. All authors have read an agreed to the publisher version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2022.113607.

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