



Levels of persistent organic pollutants in breast milk samples representing Finnish and Danish boys with and without hypospadias

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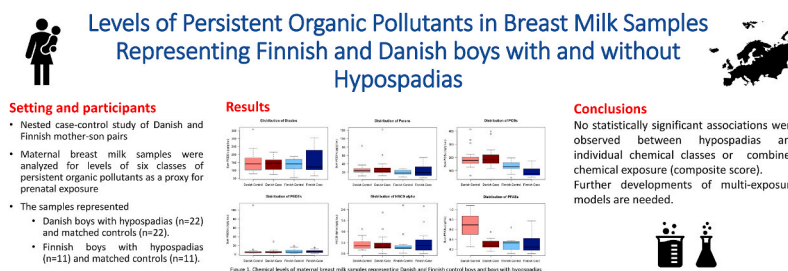
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HIGHLIGHTS

- Animal studies suggest causal links between exposure to POPs and hypospadias.
- In this human case-control study composite exposure score was also evaluated.
- No association between hypospadias and exposure to POPs or mixtures was observed.

GRAPHICAL ABSTRACT



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ABSTRACT

Hypospadias is a congenital malformation of penile urethra with unknown etiology in most cases. Persistent organic pollutant (POP) exposure may disrupt endocrine function during a critical window of development of male genitalia. In animal studies, POPs have been associated with male reproductive disorders, including hypospadias, but only few studies have assessed this relationship in humans. The aim of this study is to investigate the association between hypospadias and POP concentration levels in breast milk, as a proxy for prenatal exposure. This is a nested case-control study of Danish and Finnish mother-son pairs. Maternal breast milk samples were collected between 1997 and 2002, and they represent infant boys born with hypospadias [$n = 33$ ($n = 22$ Danish and $n = 11$ Finnish)] and their 1:1 matched controls. Breast milk samples were analyzed for six classes of POPs [including dioxins, polychlorinated biphenyls, flame retardants and perfluorinated alkylated

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substances (PFAS)]. We estimated odds ratios (ORs) and 95% confidence intervals (CI) for each chemical class using conditional logistic regression. In addition, a composite exposure score system was used to explore the effect of a POP mixture (four chemical classes): The composite score was categorized as low, moderate, or high exposure, and differences between cases and controls were tested with conditional logistic regression. No statistically significant associations were observed between the sums of the chemical classes and hypospadias in either country. The composite score was unable to detect differences in the risk of hypospadias between the tertiles of POP exposure. Levels of PFAS were significantly higher in Danish than in Finnish breast milk samples. This small study does not provide evidence for an association between hypospadias and exposure to POPs but adds information on quantitative exposures. Further development of multi-exposure models is needed for assessing the potential mixture effect associated with multiple chemical exposures.

List of abbreviations

Ah aryl hydrocarbon

BMI body mass index

dl dioxin-like

EDC endocrine disrupting chemical

GC-HRMS gas chromatography coupled to high-resolution mass spectrometry

HBCD hexabromocyclododecane

JEM job-exposure matrix

LC-HRMS liquid chromatography coupled to high-resolution mass spectrometry

LC-MS/MS liquid chromatography coupled to tandem mass spectrometry

LLE liquid/liquid extraction

LOD limit of detection

ndl non-dioxin-like

PBDE polybrominated diphenyl ethers

PCB polychlorinated biphenyls

PCDD polychlorinated dibenzo-*p*-dioxins

PCDF polychlorinated dibenzofurans

PFAS perfluorinated alkylated substances

PFOA perfluorooctanoic acid

PFOS perfluorooctanesulfonic acid

POP Persistent organic pollutant

SPE Solid Phase Extraction

WHO-TEq2005 World Health Organization Toxic Equivalent

1. Introduction

Hypospadias is a congenital malformation in males where the urethra opens on the ventral side of the penis, scrotum, or perineum, instead of the tip of the penis (van der Zanden et al., 2012). The mildest forms may not be detected until the foreskin can be retracted (Boisen et al., 2005). The mild glanular and coronal forms are the most common types found in Finnish and Danish infants (Boisen et al., 2005; Virtanen et al., 2001). The etiology of hypospadias is unknown in most human cases, but both genes and the environment are thought to have a role (Thorup et al., 2014; van der Zanden et al., 2012). Hypospadias may be caused by disruptions in balance between androgen and estrogen signaling, such as defects in biosynthesis, metabolism or action of androgens or exposure to estrogenic endocrine disrupting chemicals (EDCs) (Cripps et al., 2019; Cunha et al., 2015; Govers et al., 2019; Mattiske and Pask, 2021; Toppari et al., 2010). There is evidence that exposure to anti-androgens may be one mechanism by which penile abnormality develops (Christiansen et al., 2008; Sinclair et al., 2017). Animal studies have shown that hypospadias is common in male pups exposed in utero to chemicals inhibiting testosterone production or testosterone action via blocking the androgen receptor, such as pesticides and phthalates (Fisher et al., 2003; Gore et al., 2015; Mylchreest et al., 1999).

Many different EDCs found in the environment may have the potential to disrupt the sex hormone balance at a critical window of male genital development. EDCs include for instance persistent organic pollutants (POPs) and other chemicals, which tend to accumulate in adipose tissues and in the food chain (Bergman et al., 2013). Examples of such chemicals are dioxins, [i.e. polychlorinated dibenzo-*p*-dioxins (PCDDs) and related halogenated aromatic hydrocarbons, e.g. polychlorinated dibenzofurans (PCDFs)], polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), hexabromocyclododecane (HBCD) and perfluorinated alkylated substances (PFAS). These chemicals have the potential to disrupt sex hormone balance through different mechanisms. Dioxins, that act via the aryl hydrocarbon (Ah)

receptor, are not known to cause hypospadias on their own, but a combined disruption of the androgen and Ah receptor mediated pathways from exposure to chemical mixtures caused cumulative dose-additive effects on the development of male external genitalia (Rider et al., 2010). Dioxin-like (dl) PCBs also act via the Ah receptor, and non-dioxin-like (ndl) PCBs have anti-androgenic properties (Schrader and Cooke, 2003). PBDEs and perfluorooctanoic acid (PFOA) have been identified as having anti-androgenic potential, for at least some of their congeners (Eggert et al., 2019; Stoker et al., 2005). In utero exposure of rats to perfluorooctanesulfonic acid (PFOS) caused a reduced testosterone production and impairment of fetal Leydig cells (Zhao et al., 2014). PBDEs and HBCD are used as flame retardants. The effects of HBCD have not been well studied, though maternal serum levels of HBCD at third trimester of pregnancy showed a tendency to correlate negatively with serum free testosterone levels in their sons at the age of three months (Meijer et al., 2012). Several POPs have also estrogenic or antiestrogenic properties (De Coster and van Larebeke, 2012; Li et al., 2013). Although many POPs were banned or restricted in 2004 by the Stockholm convention or before, their ability to persist and bioaccumulate requires more research to fully understand their effects (Bergman et al., 2013).

Previous studies have shown that Danish male infants are exposed to larger concentrations of many EDCs than their Finnish counterparts (Antignac et al., 2016; Krysiak-Baltyn et al., 2010; Shen et al., 2008; Virtanen et al., 2012). Likewise, according to our joint prospective longitudinal cohort study, the prevalence of hypospadias was significantly higher in Denmark (1.03% of boys were diagnosed to have hypospadias by 3 months of age) than in Finland (0.27% of boys were diagnosed by the same age) (Boisen et al., 2005; Virtanen et al., 2001). An increasing trend of prevalence of hypospadias has been reported in many countries including Denmark (Boisen et al., 2005; Lund et al., 2009; Nissen et al., 2015), but not in Finland (Aho et al., 2000; Virtanen et al., 2001; Yu et al., 2019). In this case-control study, we examined the association between hypospadias and exposure to six groups of POPs. Levels of dioxins (PCDDs and PCDFs), PCBs, PBDEs, HBCD- α , and PFAS (PFOA and PFOS) were analyzed in the breast milk of mothers of

newborn boys in Denmark and Finland.

2. Subjects and methods

2.1. Study design and study population

This study involved the collaboration of Finnish and Danish research groups at two hospitals, Turku University Hospital and the University Hospital of Copenhagen (Rigshospitalet and Hvidovre Hospital). All the breast milk samples were collected in the Danish-Finnish prospective cohort study on the prevalence and risk factors of congenital cryptorchidism and hypospadias 1997–2002 (Boisen et al., 2004, 2005; Virtanen et al., 2001). For the current study a matched case-control design was used in both countries. Maternal breast milk chemical levels served as a proxy for prenatal exposure of newborn boys. In both countries, all hypospadias cases whose mothers succeeded in collection of sufficient amounts of breast milk sample were included in the current study. The boys were evaluated for hypospadias at birth and/or at 3 months. The standardized recruitment criteria, examination technique, and the observed prevalence rates of hypospadias have been published previously (Boisen et al., 2005; Virtanen et al., 2001). Results on chemical levels found in Finnish breast milk samples have been published previously except for PFAS, and the Danish data reported here has been published in part (Antignac et al., 2016). However, evaluation of case-control differences in maternal breast milk chemical levels between boys with and without hypospadias has not been reported before (Antignac et al., 2016).

The Danish group included $n = 44$ breast milk samples, representing 22 boys with hypospadias and 22 matched control boys. The Finnish group included $n = 22$ breast milk samples representing 11 boys with hypospadias and 11 matched controls. The matching criteria for each country was gestational age (± 7 days), maternal diabetes (yes/no), maternal smoking (yes/no), and parity, and date of birth (± 1 year).

The study was performed according to the Helsinki II declaration and was approved by the local Finnish and Danish ethics committees (KF 01-030/97/KF 01276357 in Copenhagen and 7/1996, 6/2001 in Turku) and the Danish Data Protection Agency (1997-1200-074). Oral and written informed consent was given by the parents.

2.2. Breast milk sample collection, storage, and measurements

Breast milk samples were recommended to be taken as hindmilk after feeding the child and without using breast pumps. All milk samples were collected as several small aliquots between 1- and 3-months post-partum for a sample size of 150 mL (up to a maximum of 200 mL) and frozen at each household in 250 mL Pyrex glass bottles (1515/06D; Bibby Sterilin, Staffordshire, UK) with Teflon coated caps at -20 °C until analysis.

The methodologies applied to isolate, detect, and quantify the targeted 6 POP chemical classes, including dioxins (17 PCDD/F congeners), PCBs (12 dl and 6 ndl congeners), 7 PBDE congeners, HBCD- α , and perfluoroalkylated substances (PFOA and PFOS) have been previously described (Antignac et al., 2009, 2013, 2016; Costera et al., 2006; Kadar et al., 2011), see complete list of the congeners in [Supplementary Table S1](#). Briefly, the process of PCDD/Fs, PCBs, and HBCD analysis involved first extraction, then purification, and lastly quantification. Initially, ^{13}C -labelled internal standards were added to the breast milk samples and liquid/liquid extraction was performed with pentane. By gravimetric determination, the fat content was measured from extracts and hexane was added for the subsequent purification process which involved three chromatographic steps from silica to Florisil and lastly carbon columns. PCDD/F, PCB, PBDE measurements were performed by gas chromatography coupled to high-resolution mass spectrometry (GC-HRMS) and HBCD measurements were performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Whereas the semi-persistent PFAS compounds were analyzed by a separate method described elsewhere (Kadar et al., 2011). This included

a liquid/liquid extraction (LLE) for preliminary protein precipitation followed by two stages of purification by Solid Phase Extraction (SPE) first in Oasis® HLB and then carbon graphitized (Evanicarb®) cartridges. The extract was reconstituted in 200 μL of a fluorometholone solution as external standard in a methanol/water mixture (30/70 v/v). PFAS measurements were performed by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS).

These procedures were validated according to current European standards in the field of regular control of foodstuff of animal origin and the ISO 17025 standard. Quality Assurance/Quality Control (QA/QC) procedures included systematic analysis of negative (blank, $n = 3$) and positive (standard reference material, $n = 1$) control samples in all batches of analyzed samples, and several interlaboratory assays were completed at national and international level to confirm the method robustness and accuracy.

2.3. Statistics

Population characteristics are given as numbers or medians and ranges. Differences between boys with and without hypospadias were analyzed by country of origin with the Wilcoxon-Signed Rank test. As the samples were matched on gestational age, maternal diabetes, maternal smoking, and parity, the case-control differences were not tested for these variables.

In order to control for the time trends in exposure to POPs during the study period 1997–2002, a variable was created where each child was ranked by birth date and year by setting the first child born in this study to zero, and each child thereafter was ranked by the number of days that had elapsed since the first child's birthdate. This was considered the variable "rank of birthdate". This was calculated within each country and also for the two countries combined.

Conditional logistic regression was used when estimating the magnitude of associations between exposure to POPs and hypospadias in Finland and Denmark separately and combined. The sums of the congeners in each of 6 chemical classes [PCDDs, PCDFs, PCBs (seven indicator PCBs included: ndl congeners PCB-28, -52, -101, -138, -153, -180 and dl congener PCB-118), PBDEs, α -HBCD, and PFAS] were analyzed as independent continuous variables. This analysis also included the following subgroups: WHO-TEQ₂₀₀₅ PCDD/F, WHO-TEQ₂₀₀₅ dl-PCB, Total WHO-TEQ₂₀₀₅, coplanar PCBs, and non-coplanar PCBs. When the chemical measurement was below the lower limit of detection (LOD) the value was set as zero (lower bound approach). When evaluating for case-control differences, a stepwise conditional logistic regression with backward elimination was completed. Maternal body mass index (BMI), maternal age, weight for gestational age, and rank of birthdate in the cohort were included in the full model. Although not significant, the final adjusted regression model included only maternal BMI and maternal age. Multicollinearity was assessed for all continuous predictors and the correlations between exposure levels and other predictors were evaluated with Spearman's rho and Pearson's r . All analyses used untransformed data.

To assess the degree of exposure to mixtures of POPs a scoring system was created based on another poly-exposure classification model (Brucker-Davis et al., 2008). For each country, the sum of each chemical class for which 100% of samples were analyzed (PCDDs, PCDFs, PCBs, PBDEs) was divided into tertiles and scored - 1 was assigned for the first tertile, 2 for the second tertile, and 3 for the third tertile. The composite score for each subject (case or control) was calculated by adding the individual score numbers of the 4 chemical classes. The composite scores of all subjects were then divided into tertiles for low, moderate, and high exposure to the mixture. Differences in exposure levels between cases and controls were evaluated by country and also countries combined with simple conditional logistic regression using the low exposure as the reference group.

Differences in breast milk PFAS levels between Finland and Denmark were explored using Mann-Whitney-U test. The criterion for statistical

significance was a two-tailed p-value of <0.05. Correction for multiple comparisons testing was not applied. All statistics were performed using R and RStudio (v.3.3.2).

3. Results

The range of severity of cases with hypospadias consisted of 1 penoscrotal case, 1 coronal/penile case, and 31 glanular or coronal (mild) cases. Five of the cases had also congenital cryptorchidism. Three of the hypospadias cases had a first degree relative with hypospadias. Two hypospadias cases (one from Finland and one from Denmark) had a brother with hypospadias and one Danish case had a father with hypospadias. There were no significant differences between the cases and controls in maternal age, BMI, weight for gestational age, or birth weight in either country (Table 1) or combined (data not shown).

The chemical concentrations in the case and control groups are shown in Table 2. There were no significant differences in exposure levels between the cases and controls analyzed for the countries combined or separated (Fig. 1 and Supplementary Tables S2 and S3, respectively). There was no significant difference between cases and controls for the PCB congeners with the highest concentrations: PCB-118, -138, -153, -156, and -180 (Supplementary Table S4).

The composite score for the mixture of exposures is shown in Table 3. There was no difference between cases and controls when analyzing tertiles of composite exposure levels (countries combined or separately).

When comparing the Danish and Finnish data for their country-specific chemical signature, the sum of PFAS was significantly higher in Danish than in Finnish breast milk samples, median (range) 0.47 (0.16–1.08) ng/mL vs. 0.28 (0.11–0.77) ng/mL, respectively, $p = 0.002$.

4. Discussion

The hypothesis that prenatal exposure to POPs is associated with an increased risk of hypospadias (Skakkebaek et al., 2016) was not supported by our study. There was no association seen with individual chemical classes, or with combined chemical exposure (composite score). Human exposure to environmental chemicals occurs rarely in isolation and therefore evaluation of effects of chemical exposure using a composite score was done. This allowed us to consider the differences in the magnitude of chemical exposure between the two countries, with Finland having a significantly lower burden of exposure than Denmark for many POPs.

Human studies investigating the association between hypospadias and early exposure to chemicals are scarce, and of those, few studies use quantitative measurements from human tissues. This study adds to the epidemiologic evidence by including individual measurements of chemical levels in human breast milk. Many epidemiologic studies rely

instead on retrospective surveys, questionnaires, interviews about past exposure through job-exposure matrices (JEMs) and exposure estimates based on residential information. Thus, they may be prone to misclassification bias. Some, but not all, studies have found associations between maternal residential exposure to EDCs (Agopian et al., 2013; Bougnères et al., 2021; Carmichael et al., 2013; Cognez et al., 2019; García et al., 2017; Meyer et al., 2006; Rappazzo et al., 2016; Sheth et al., 2019; Winston et al., 2016) or maternal and paternal job exposures to EDCs (especially to pesticides) and hypospadias (Estors Sastre et al., 2019; Giordano et al., 2010; Haraux et al., 2016; Kalfa et al., 2015; Morales-Surez-Varela et al., 2011; Nassar et al., 2010; Rocheleau et al., 2009, 2011; Vrijheid et al., 2003).

Breast milk chemical levels were used as a proxy for fetal exposure in our study (Damgaard et al., 2006). Other studies have investigated exposure to POPs, including additional chemicals that were not included in our analysis, by analyzing chemical levels in boys' serum, maternal serum, amniotic fluid or hair samples (Bhatia et al., 2005; Carmichael et al., 2010; Giordano et al., 2010; Koren et al., 2019; Longnecker et al., 2002; McGlynn et al., 2009; Poon et al., 2018; Rignell-Hydbom et al., 2012; Shekharyadav et al., 2011; Small et al., 2009; Toft et al., 2016; Trabert et al., 2012). Boys' serum levels of the pesticide hexachlorohexane and DDT metabolite DDE were significantly increased in hypospadias cases compared to controls in one study (Shekharyadav et al., 2011). High maternal serum levels of hexachlorobenzene were associated with increased risk of hypospadias in two studies (Giordano et al., 2010; Rignell-Hydbom et al., 2012). However, other studies evaluating maternal serum levels of pesticides, PCBs and flame retardants (PBDEs and polybrominated biphenyls) found no significant association with hypospadias or the results were inconsistent (Bhatia et al., 2005; Carmichael et al., 2010; Longnecker et al., 2002; McGlynn et al., 2009; Small et al., 2009; Trabert et al., 2012). Our study found no significant association between either PCB or PBDE exposure and hypospadias. However, a larger study evaluating PBDE levels in maternal hair samples reported significantly higher levels among mothers of infants with hypospadias ($n = 152$) as compared to controls ($n = 64$) (Koren et al., 2019; Poon et al., 2018). We found no significant association between PFAS exposure and hypospadias, which is in line with a previous study analyzing PFOS levels in amniotic fluid (Toft et al., 2016). We found no significant association between hypospadias and dioxin or HBCD- α exposure. To our knowledge, there are no previous studies evaluating these compounds.

Our previous studies reported higher chemical levels in Danish than in Finnish breast milk, cord blood, and whole placenta samples (Antignac et al., 2016; Jensen et al., 2014; Krysiak-Baltyn et al., 2010; Main et al., 2007; Virtanen et al., 2012) for many, but not all chemical groups presented here. We observed significantly higher PFAS levels in Danish milk samples as compared to Finnish milk samples. Country differences

Table 1

Population characteristics [median (range) or numbers] for Danish and Finnish mothers and infant boys.

	Denmark			Finland		
	Control (n = 22)	Hypospadias (n = 22)	p-value	Control (n = 11)	Hypospadias (n = 11)	p-value
Maternal age (years)	29.8 (23.6–36.3)	30.1 (22.9–36.6)	1.00	32.0 (27.4–44.7)	30.9 (23.8–39.6)	0.46
BMI (kg/m ²) ^a	22.4 (19.4–29.3)	20.8 (17.1–40.6)	0.12	21.2 (18.7–27.2)	23.3 (20.1–26.2)	0.10
Diabetes (yes/no)	0/22	0/22	NA	0/11	0/11	NA
Smoking (Y/N) ^b	4/17	5/17	NA	1/10	1/10	NA
Parity (no.)						
1	18	18	NA	5	4	NA
2	3	3		5	5	
≥ 3	1	1		1	2	
Gestational age (days)	286 (256–294)	285 (249–296)	NA	279 (263–288)	276 (249–303)	NA
Weight for gest. age (%)	3.0 (–10.0 - 15.1)	–4.4 (–31.0 - 44.4)	0.19	–1.5 (–11.0 - 23.0)	4.5 (–30.8 - 38.3)	1.00
Birth weight (kg)	3.8 (3.0–4.3)	3.6 (1.9–4.8)	0.14	3.5 (3.0–4.6)	3.6 (2.0–4.7)	1.00

Wilcoxon-Signed Rank test; BMI, body mass index; NA, not applicable due to matched design.

^a Maternal BMI data is missing for 5 Danish mothers and 4 Finnish mothers.

^b Smoking data is missing for 1 Danish mother.

Table 2
Breast milk chemical concentrations and odd ratios for the association with hypospadias.

Countries Combined												
Chemical Group ^a	Unit	Control (n = 33)			Hypospadias (n = 33)			Crude ^b		Adjusted ^c		
		DF (%)	n	Median (range) ^a	DF (%)	n	Median (range) ^a	p-value	OR (95% CI)	p-value	OR (95% CI)	
Σ PCDD	pg/g l. w.	100	33	140.60 (55.10–358.10)	100	33	140.80 (66.85–305.70)	0.93	1.00 (0.99–1.01)	0.90	1.00 (0.99–1.01)	
Σ PCDF	pg/g l. w.	100	33	22.56 (9.23–83.09)	100	33	21.71 (7.11–121.90)	0.41	1.02 (0.98–1.05)	0.79	1.01 (0.96–1.06)	
WHO-TEQ ₂₀₀₅ PCDD/F	pg/g l. w.	100	33	12.86 (3.93–29.50)	100	33	12.33 (3.48–38.59)	0.94	1.00 (0.91–1.10)	0.89	0.99 (0.86–1.14)	
Σ PCB ^d	ng/g l. w.	100	33	165.00 (47.72–413.80)	100	33	158.30 (52.35–397.90)	0.53	0.99 (0.99–1.01)	0.53	0.99 (0.99–1.01)	
Σ Coplanar PCB ^e	pg/g l. w.	100	33	90.70 (32.33–188.00)	100	33	89.09 (28.40–229.80)	0.45	1.01 (0.99–1.02)	0.38	1.01 (0.99–1.03)	
Σ Non-coplanar PCB ^f	pg/g l. w.	100	33	25,550 (7297–60,420)	100	33	24,490 (7719–59,990)	0.57	1.00 (0.99 ^h - 1.00)	0.66	1.00 (0.99 ^h - 1.00)	
WHO-TEQ ₂₀₀₅ dl-PCB	pg/g l. w.	100	33	6.65 (2.28–13.56)	100	33	6.53 (2.01–16.41)	0.66	1.04 (0.86–1.26)	0.70	1.04 (0.83–1.31)	
Total WHO-TEQ ₂₀₀₅	pg/g l. w.	100	33	19.68 (6.21–42.93)	100	33	18.08 (5.72–46.24)	0.82	1.01 (0.94–1.08)	0.95	1.00 (0.92–1.10)	
Σ PBDE	ng/g l. w.	100	33	4.75 (2.16–111.10)	100	33	5.24 (1.47–28.91)	0.49	0.98 (0.94–1.03)	0.40	0.93 (0.79–1.10)	
α -HBCD ^g	ng/g l. w.	100	32	0.32 (0.03–2.19)	94	33	0.31 (0.0–2.15)	0.69	1.36 (0.30–6.15)	0.72	1.35 (0.26–7.14)	
Σ PFAS	ng/mL	100	27	0.48 (0.12–1.08)	100	23	0.27 (0.11–0.77)	0.06	0.03 (<0.001–1.10)	0.08	0.01 (<0.001 to 1.70)	

DF, detection frequency of chemicals in breast milk samples; OR, odds ratio; CI, confidence interval; PCDD/PCDF, polychlorinated dibenzo-*p*-dioxins and dibenzofurans; WHO-TEq, World Health Organization Toxic Equivalent; PCB, polychlorinated biphenyls; dl: dioxin-like; l.w., lipid weight; PBDE, polybrominated diphenyl ethers; HBCD, hexabromocyclododecane; PFAS, perfluorinated alkylated substances.

^a Untransformed chemical data.

^b Conditional logistic regression model.

^c Conditional logistic regression model adjusted for maternal age and maternal BMI, body mass index.

^d Sum of PCB-28, -52, -101, -118, -138, -153, -180 ^ecoplanar PCB-77, -81, 126, -169.

^f Noncoplanar PCB-105, -114, -118, -123, -156, -157, -167, -189.

^g One outlier removed.

^h 0.99999.

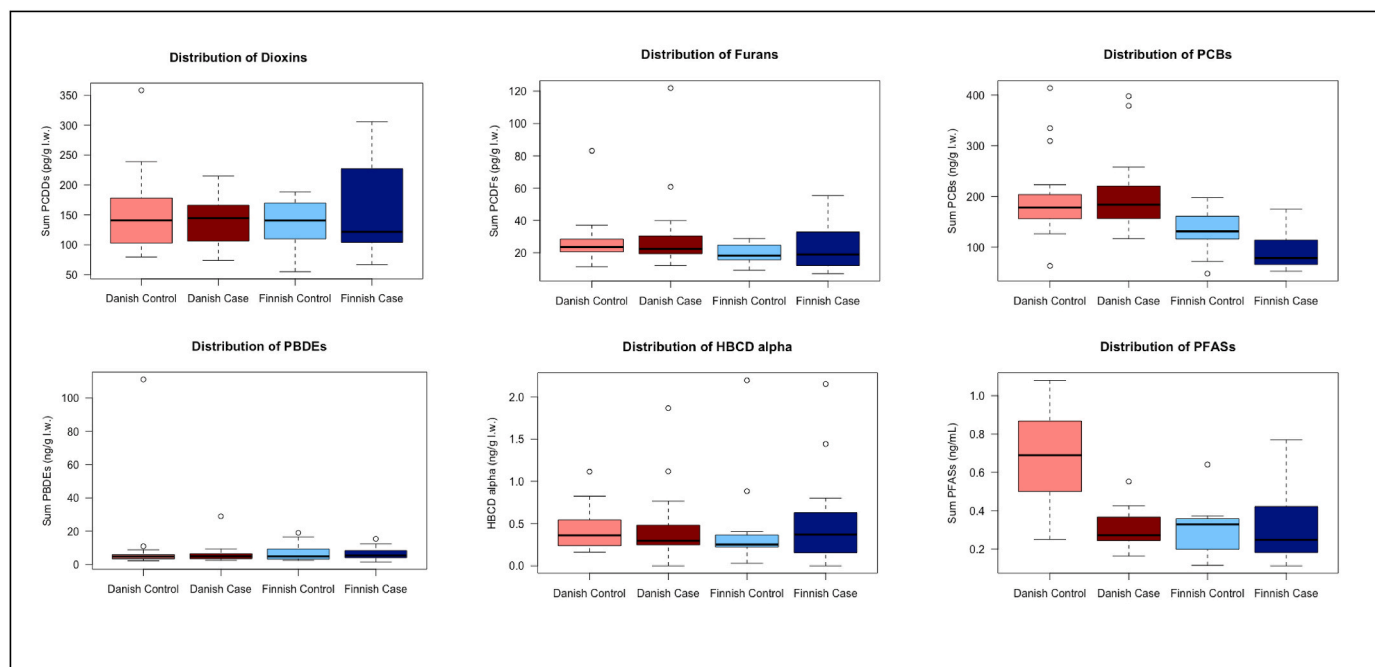


Fig. 1. Distributions of levels of persistent organic pollutants (sum of PCDDs, PCDFs, PCBs, PBDEs, PFASs and HBCD- α) in breast milk samples representing Danish and Finnish control boys and boys with hypospadias. (PCDDs, polychlorinated dibenzo-*p*-dioxins; PCDFs, polychlorinated dibenzofurans; PCBs, polychlorinated biphenyls; PBDEs, polybrominated diphenyl ethers; HBCD- α , hexabromocyclododecane alpha; PFASs, perfluorinated alkylated substances).

Table 3
Composite scores of exposure and odds ratios for the associations with hypospadias.

Countries Combined Composite Score						
Composite Score	Score	Total	Controls	Hypospadias	p-value ^a	OR (95% CI)
4 chemical classes ^b	low (4-6)	23	10	13	reference	reference
	moderate (7-9)	26	16	10	0.27	0.49 (0.14–1.75)
	high (10-12)	17	7	10	0.83	0.85 (0.20–3.63)
Danish Composite Score						
Composite Score	Score	Total	Controls	Hypospadias	p-value ^a	OR (95% CI)
4 chemical classes ^b	low (4-6)	15	6	9	reference	reference
	moderate (7-9)	17	11	6	0.27	0.48 (0.13–1.75)
	high (10-12)	12	5	7	0.90	0.90 (0.19–4.28)
Finnish Composite Score						
Composite Score	Score	Total	Controls	Hypospadias	p-value ^a	OR (95% CI)
4 chemical classes ^b	low (4-6)	8	4	4	reference	reference
	moderate (7-9)	9	5	4	0.66	0.67 (0.11–3.99)
	high (10-12)	5	2	3	NA	NA

OR, odds ratio; CI, confidence interval; NA not applicable because not accurately estimated.

For each country, the sum of each chemical class for which 100% of samples were analyzed (PCDDs, PCDFs, PCBs, PBDEs) was divided into tertiles and scored: 1 was assigned for the first tertile, 2 for the second tertile, and 3 for the third tertile. The composite score for each subject was obtained by summing up the individual score numbers of the 4 chemical classes (range 4–12). The composite scores of all subjects were then divided into tertiles for low, moderate, and high exposure.

^a Simple conditional logistic regression.

^b Chemical groups include: ΣPCDD, ΣPCDF, ΣPCB, ΣPBDE.

in exposure to PFASs were also observed in cord blood samples during this time period (1997–2002), with Denmark having an approximately two-fold higher exposure than Finland (Jensen et al., 2014).

Hypospadias is a rare condition and it is therefore difficult to collect enough cases to reach statistical power within longitudinal cohort studies (Skakkebaek et al., 2016). Many studies that found non-significant results often also had small samples sizes and/or they included cases of hypospadias combined with cases of cryptorchidism. Our study represents a small dataset (33 cases) and may thus be underpowered to show a true difference between cases and controls. In addition, we cannot exclude some variation in timing of breast milk sample collection (collection time up to two months), which may affect chemical levels (Thomsen et al., 2010). Furthermore, POPs have been regulated in the past several decades and subsequently, their production and release has declined as observed by time trends in air and human tissue samples (Hardell et al., 2010; Hung et al., 2016), which may have reduced our potential to find a significant association.

5. Conclusions

In conclusion, our study found no evidence for an association between hypospadias and prenatal exposure to POPs for individual chemical groups or a composite exposure score for a mixture of PCDDs, PCDFs, PCBs and PBDEs. Further developments of multi-exposure models are needed for assessing the potential mixture effect associated to the multiple chemical exposures.

Credit author statement

Marie Tysman: Formal analysis, Writing – original draft, review & editing. Jorma Toppari: Conceptualization, Formal analysis, Writing – review & editing. Katharina M Main: Conceptualization, Investigation, Writing – review & editing. Annika Adamsson: Investigation, Formal analysis, Writing – original draft, review & editing. Christine Wohlfahrt-Veje: Investigation, Project administration, Writing – review & editing. Jean-Philippe Antignac: Investigation, Methodology, Writing – review & editing. Bruno Le Bizec: Conceptualization, Methodology, Investigation, Writing – review & editing. Eliisa Löytyniemi: Formal analysis, Writing – review & editing. Niels E Skakkebaek: Conceptualization, Writing – review & editing. Helena E Virtanen: Investigation, Formal analysis,

Project administration, Writing – original draft, review & editing.

Data statement

The dataset supporting the conclusions of this article comprises health related participant data and their use is therefore restricted under the regulations on professional secrecy (Act on the Openness of Government Activities, 612/1999) and on sensitive personal data (Personal Data Act, 523/1999, implementing the EU data protection directive 95/46/EC). Due to these legal restrictions, the data from this study cannot be stored in public repositories or otherwise made publicly available. However, data access may be permitted on a case by case basis upon request only. Data sharing outside the research groups is done in collaboration with the research groups and requires a group-specific data transfer agreements (DTA). Investigators can submit an expression of interest to the chairman of the Finnish steering group (Prof Jorma Toppari, University of Turku, Turku, Finland. Email: jorma.toppa@utu.fi).

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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.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2022.137343>.

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