Ecotoxicology and Public Health

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Environ. Sci. Technol., **Just Accepted Manuscript** • DOI: 10.1021/acs.est.9b06054 • Publication Date (Web): 21 Dec 2019

Persistent Organic Pollutants in Human Breast Milk and Associations with Maternal Thyroid Hormone Homeostasis

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ABSTRACT

Epidemiological studies have indicated the thyroid-disrupting effects of persistent organic pollutants (POPs). However, the associations of low-exposure POPs with thyroid hormones (THs) remain unclear. Here we aim to assess the associations of low exposure of POPs, including polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), and polybrominated dibenzo-*p*-dioxins and furans (PBDD/Fs), with THs (total $_L$-thyroxine (TT_4), total 3,3',5-triiodo-$_L$-thyronine (TT_3), and total 3,3',5'-triiodo-$_L$-thyronine (TrT_3)) measured in human breast milk. Ninety-nine breast milk samples were collected from the LUPE cohort (2015–2016, Bavaria, Germany). Fourteen PBDEs, 17 PCBs, and 5 PCDD/Fs had quantification rates of > 80%. Nonmonotonic associations were observed. In adjusted single-pollutant models: (1) TT_4 was inversely associated with BDE-99, -154, and -196; (2) TT_3 was inversely associated with BDE-47, -99, -100, -197, -203, -207, and OCDD; (3) TrT_3 was inversely associated with BDE-47, -99, -183, and -203. Multipollutant analysis using principal component analysis and hierarchical clustering revealed inverse associations of PBDEs (BDE-28, -47, -99, -100, -154, -183, and -197) with TT_4 and TrT_3. These results indicate that POPs at low levels might be related to reduced THs. This study shows that human breast milk might be an appropriate specimen to evaluate the thyroid-disruption of POPs.

1. Introduction

Persistent organic pollutants (POPs) are a group of chemicals with environmental persistence, bioaccumulation, and toxicity. They occur as a result of industrial and commercial applications, incomplete incineration, traffic, and industrial processes [1]. Common POPs include polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), and polybrominated dibenzo-*p*-dioxins and furans (PBDD/Fs). Humans are exposed to POPs through diet, air, house dust, and drinking water. Although many POP congeners have been strictly limited or banned, exposure to these compounds continues because of their long half-lives [2]. POPs have been detected in the environment and humans all over the world [3, 4].

Certain POPs have chemical structures similar to thyroid hormones (THs) leading to concerns about their potential of thyroid disruption. TH homeostasis is crucial for down-stream physiological processes such as metabolism, growth, bone remodeling, cardiac function and mental status [5]. POPs can interact with any aspect of the hypothalamus-pituitary-thyroid (HPT) axis, TH biosynthesis, metabolism, and release, feedback regulation, transport, agonist or antagonist thyroid hormone receptor (TR), and regulation of uridine diphosphate glucuronosyltransferases (UDPGTs) and sulfotransferases (SULTs) [5-7]. POP exposures may partially contribute to the rapid increasing incidence of thyroid diseases such as hypothyroidism, hyperthyroidism, and thyroid cancer [8-10].

In vitro and animal studies have proved TH disruption following POP exposures [11, 12]. Human studies also observed associations between THs and POPs using peripheral/cord blood [13-16] and placenta [17, 18]. Conflicting results regarding the direction of associations have been reported [15, 19-21]. Possible reasons include the low-dose effects and non-monotonic effects of endocrine-

disrupting chemicals (EDCs) [22]. For example, positive association between PBDEs and THs was found in a high-exposure population (median ΣPBDEs: 38.4 ng/g lipid weight (lw)) [21], whereas negative association was reported in a low-exposure population (median ΣPBDEs: 3.49 ng/g lw) [23]. The thyroid-disrupting effects of POPs at low levels are of concern since most of the current studies were conducted in high-exposure populations. However, the detection of POPs in blood of low-exposure population requires high sensitivity or large sample volume to obtain sufficient detection frequencies (DFs) [24], which can be limiting for certain age groups.

Human breast milk is a complex and constantly changing mixture of endogenous and exogenous substances including THs and POPs [3, 25]. Due to its high lipid content, breast milk has been considered as an appropriate specimen to provide improved sensitivity for POP monitoring [3, 26]. Besides, the serum TH homeostasis may be evaluated by examining THs in breast milk because of the significant positive correlations between milk THs and serum THs [27]. Several studies have assessed the associations between POPs in milk and serum TH parameters [28, 29]. Darnerud *et* al. found that low chlorinated PCBs in breast milk were inversely associated with total 3,3',5-triiodo-$_L$-thyronine (TT_3) in serum of 3-week old children, while PCDD/Fs in breast milk showed negative associations with maternal serum TT_3 [29]. However, no study has been conducted to evaluate the associations of THs with POPs both measured in human breast milk.

The primary goal of the current study was to evaluate the associations of POPs (PBDEs, PCBs, PCDD/Fs, and PBDD/Fs) with THs (total $_L$-thyroxine (TT_4), TT_3, and total 3,3',5'-triiodo-$_L$-thyronine (TrT_3)) measured in human breast milk. Samples were collected from the LUPE cohort (2015–2016, Bavaria, Germany), which is exposed to low levels of POPs from a global perspective. Single-pollutant and multipollutant models were applied to evaluate the relationship between THs and POPs.

2. Materials and methods

2.1 Sample collection

We included 99 human breast milk samples in this study. Approximately 150 mL of sample was collected from each participating woman within 10 months after delivery. Samples were collected into sample cups (AVENT VIA) using a manual breast pump (AVENT ISIS) after breastfeeding. Afterwards, samples were transported to the Bavarian Health and Food Safety Authority (Munich, Germany) for POP determination. An aliquot of 2 mL was delivered to the Helmholtz Center Munich (Munich, Germany) for TH analysis. Samples were stored at -80 ℃ until processing.

The ethics committee of the Bavarian Chamber of Physician approved this study. Informed written consent was obtained from each participant.

2.2 POP analysis

Detailed analytical methods regarding POP quantificaiton are available elsewhere [30, 31]. The materials are shown in the Supporting Method. Briefly, milk lipid was extracted with *n*-hexane/propane-2-ol and applied on a column composed of Isolute HM-N/sodium chloride. The concentrated lipid extract was dried on an anhydrous sodium sulphate column and extracted with *n*-pentane. After further automated clean-up and fractionation with DEXTech (3 columns setup), the final extracts were analyzed by two gas chromatographs/high resolution mass spectrometer (2GC/HRMS) on a Thermo DFS system with three different columns. The World Health Organization Toxicant Equivalent Quotient (WHO_{2005}-TEQ) of dioxins and dioxin-like PCBs (dl-PCBs) was calculated [32]. The average method quantification limits (MQLs) were 0.125 pg/g lw for PCDD/Fs, 2.63 pg/g lw for dl-PCBs, 4.71 pg/g lw for non-dl-PCBs, 4.16 pg/g lw for PBDD/Fs, and 3.99 pg/g lw for PBDEs. The recoveries of these POPs ranged overall from 50% to 140% and comply with the requirements of Regulation (EU) No. 589/2014.

2.3 TH measurement

Total levels of T_4, T_3, rT_3, 3,3'-diiodo-$_L$-thyronine (3,3'-T_2), 3,5-diiodo-$_L$-thyronine (3,5-T_2), 3-iodo-$_L$-thyronine (T_1) and 3-iodothyronamine (3-T_1AM) were targeted for analysis in breast milk using isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS). The method was based on our previous technology with some modifications [33]. Complete details can be found in the Supporting Method, Table S1, and Fig. S1-2. The method detection limits (MDLs) and MQLs were 0.01–0.13 ng/mL and 0.10–0.42 ng/mL, respectively. The matrix effects were between -9.67% and 14.7%. The overall recoveries ranged from 102% to 125%. The spike-recoveries were in the range of 98.4%–122%. The intra-day and inter-day variations were 0.47%–6.91% and 1.37%–7.71%, respectively (Table S2).

2.4 Statistics

The statistical analyses were conducted on POP congeners with DF of > 80%, measurements below the LOQ were replaced by LOQ × DF [34]. Normality was tested using Shapiro-Wilk test. The distributions of biomarkers were log-normal and therefore transformed by the natural logarithm. We examined the bivariate associations between biomarkers and a set of demographic variables using t-test or analysis of variance (ANOVA). Afterwards, Spearman's rank correlation was applied to evaluate the correlation of biomarkers. Statistical analyses were conducted using R (version 3.4.2; R Foundation for Statistical Computing, Vienna, Austria) and DAGitty v2.3 [35] for constructing directed acyclic graph (DAG). Statistical significance was defined as p-value < 0.05.

Potential confounders considered for inclusion in models were maternal age, educational level, parity, smoking, diet, infant gender, infant age at sampling. Data on most covariates were complete. Confounders were identified based on previous reports and a DAG framework (Fig. S3).

Body mass index (BMI) was not controlled because BMI might be a consequence of thyroid dysfunction [36, 37].

Single-pollutant models were conducted to investigate the associations between THs and each POP congener. Generalized additive models (GAM) were used to examine the linearity of the relationship between POPs and THs. Some of the POP congeners showed significant non-linear associations with THs (data not shown), thus we modeled all exposure biomarkers in categories defined by tertiles.

Principal component analysis

Due to the structural and biological similarity within and across the classes, interpretation of the effect of individual POP congeners can be misleading. We assessed the multiple collinearity by the eigen values of the correlation and the variable inflation factor (VIF). Principal component analysis (PCA) was then conducted to convert the correlated variables into a small number of principal components (PCs). Afterwards, varimax rotation was applied to calculate factor scores for each participant. The number of factors was decided based on the scree plot [38]. The factor scores were categorized into tertiles and included in the regression models. Regressions were performed including factors simultaneously and separately.

Hierarchical clustering

We used the partial least squares (PLS) regression to evaluate the impact of all POPs and covariates on THs simultaneously. Only variables with variable importance to projection (VIP) values > 0.4 were included in the final model to reduce data and increase the model predictive ability [14]. The score of each participant on PC1 was included in multiple linear regression models as a common vector to avoid collinearity while adjusting for these factors. In order to minimize the number of POPs to be included in linear regression models, we conducted hierarchical

clustering analysis of POPs based on correlations (method: complete linkage). Groupings according to clusters were subsequently performed by simple addition of POP concentrations.

Sensitivity analyses

Previous studies measuring serum POPs typically adjusted for lipid content. However, there is controversy regarding the best approach [39]. In this study we performed the analyses including POPs in units of ng/g lipid. In sensitivity analysis we repeated the analyses with POPs in units of ng/L milk while controlling for lipid content as a covariate. Additional sensitivity analysis included the adjustment of BMI.

3. Results

3.1 Biomarker concentrations and their correlations

An LC-MS/MS method was optimized and validated for TH quantification in human breast milk. The mean ± SD concentrations of TT_4, TT_3, and TrT_3 were 0.57 ± 0.20, 0.13 ± 0.03, and 0.02 ± 0.01 ng/mL, respectively (Table S3).

As shown in Tables 1 & S4, 14 PBDEs had DFs of > 80%. The median concentrations of these PBDEs were 8.25–440 pg/g lw, in which BDE-209 was the dominating congener, followed by BDE-153, -47, -197, -99, -207, -100, -28, -206, -183, -208, -196, -203, and -154. Seventeen PCBs were detected in > 80% of the samples with median concentrations in the range of 0.14–3619 pg/g lw. The dominating congener was PCB-118 followed by PCB-156, -167, -105, -157, -114, -189, -123, -153, -126, -138, -169, -180, -77, -28, -101, and -52. Five PCDD/Fs were quantified in > 80% of the samples with median concentrations between 1.03 and 17.0 pg/g lw. The dominating congener was OCDD followed by 2,3,4,7,8-PeCDF, 1,2,3,6,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD, and 1,2,3,6,7,8-HxCDF. The DFs of all the PBDD/Fs were ≤ 38% and therefore not included in the statistical analyses. As shown in Table S5, the highest WHO_{2005}-TEQ levels in dl-PCBs and

PCDD/Fs were found for PCB-126 (1.81 pg/g lw) and 2,3,4,7,8-PeCDF (1.12 pg/g lw), respectively. The median values of Σmono-ortho PCBs, Σnon-ortho PCBs, ΣPCBs, ΣPCDD/Fs, ΣPBDD/Fs, and ΣPOPs were 2.78, 0.33, 3.11, 4.37, 0.93, and 8.22 pg/g lw, respectively. Table S6-S8 show the comparison of POP levels in human breast milk reported here with recent studies from different regions. POP levels in this study were generally lower, especially compared with those measured in North America.

Table 1 Descriptive statistics of PBDEs, PCBs, PCDD/Fs, and PBDD/Fs with DFs > 80% in human breast milk from LUPE study (2015–2016, Bavaria, Germany).

POPs	N (%)	Mean (pg/g lw)	Range (pg/g lw)	Q1 (pg/g lw)	Median (pg/g lw)	Q3 (pg/g lw)
BDE-28	95 (96)	31.7	<LOQ–122	20.2	29.3	37.7
BDE-47	99 (100)	307	61.6-2419	136	204	299
BDE-99	98 (99)	85.8	<LOQ-419	46.1	62.5	93.9
BDE-100	97 (98)	73.7	<LOQ–364	31.3	54.3	92.5
BDE-153	99 (100)	460	112–1979	304	377	545
BDE-154	82 (83)	9.42	<LOQ–28.5	5.58	8.25	11.1
BDE-183	97 (98)	33.7	<LOQ–182	19.4	28.4	42.2
BDE-196	89 (90)	22.4	<LOQ–146	12.4	16.8	24.1
BDE-197	99 (100)	83.2	19.3–224	53.6	73.1	103
BDE-203	91 (92)	22.8	<LOQ–265	12.8	16.7	24.4
BDE-206	86 (87)	176	<LOQ–3545	19.1	28.9	57.3
BDE-207	98 (99)	147	<LOQ–2842	39.7	56.3	82.0
BDE-208	98 (99)	67.5	<LOQ–1540	13.3	19.3	34.0
BDE-209	95 (96)	4444	<LOQ–104000	287	440	1074
PCB-28	99 (100)	0.99	0.24–4.36	0.61	0.81	1.16

PCB-52	99 (100)	0.18	0.06–1.62	0.11	0.14	0.19
PCB-77	82 (83)	3.55	<LOQ–17.2	2.37	2.97	3.83
PCB-101	99 (100)	0.37	0.10–6.20	0.18	0.25	0.34
PCB-105	99 (100)	666	177–2067	459	591	785
PCB-114	99 (100)	251	54.8–810	159	224	330
PCB-118	99 (100)	3955	1044–9635	2821	3619	4881
PCB-123	97 (98)	42.5	<LOQ–110	28.7	37.6	55.1
PCB-126	99 (100)	20.1	4.40–58.5	14.1	18.1	23.5
PCB-138	99 (100)	15.2	3.93–35.6	10.1	14.0	18.2
PCB-153	99 (100)	26.3	5.76–70.0	17.6	23.3	34.7
PCB-156	99 (100)	2668	526–8664	1633	2128	3573
PCB-157	99 (100)	393	78.3–1143	241	344	528
PCB-167	99 (100)	688	157–1481	447	662	877
PCB-169	98 (99)	12.6	<LOQ–34.1	7.88	10.9	15.2
PCB-180	99 (100)	15.7	2.69–91.8	8.76	13.2	21.3
PCB-189	99 (100)	251	36.1–1339	133	219	351
1,2,3,6,7,8-HxCDD	87 (88)	3.26	<LOQ–14.2	2.22	2.94	3.99
1,2,3,4,6,7,8-HpCDD	91 (92)	3.26	<LOQ–14.7	1.96	2.62	3.89
OCDD	98 (99)	21.2	<LOQ–75.0	12.9	17.0	24.4
2,3,4,7,8-PeCDF	94 (95)	4.21	<LOQ–10.8	3.01	3.80	5.15
1,2,3,6,7,8-HxCDF	80 (81)	1.18	<LOQ–7.86	0.72	1.03	1.41
ΣPBDEs	99 (100)	5753	511–112998	1123	1731	2727
ΣPCBs	99 (100)	9090	2222–20225	6238	8322	11211
ΣPCDD/Fs	99 (100)	35.7	0.00–115	24.3	30.2	41.6

ΣPBDD/Fs	99 (100)	43.2	0.00–1352	0.00	4.53	11.5

Abbreviations: LOQ, limit of quantification. lw, lipid weight. Q1, Q3: first and third quantile.

As shown in Fig. S4, THs showed weak negative to weak positive correlations with most of the POPs (T_4: -0.25–0.17, T_3: -0.34–0.01, rT_3: -0.28–0.11). The intragroup correlations of PBDEs, PCBs, and PCDD/Fs were -0.05–0.90, 0.0001–0.98, and 0.18–0.68, respectively.

3.2 Population characteristics

Table S9 summarizes the sociodemographic characteristics of all the participants. The mean ± SD age was 33.9 ± 4.4 years. Among them, 84 (84.8%) of them were > 30 years old; 66 (66.7%) had a BMI value of < 25 kg/m^2; majority (95.0%) did not smoke; 45 (45.5%) were nullipara. The mean ± SD infant age at sampling was 114 ± 57 days. As shown in Tables S9-S12, we observed significant correlations between demographic variables and biomarkers.

3.3 Single-pollutant model

As shown in Fig. 1 and Table S13, single-pollutant, crude models for the 36 POPs showed a significant decrease in TT_4 with increasing exposure to BDE-99, -154, -169, -196, -203, PCB-169, and 1,2,3,6,7,8-HxCDD. After adjustment, TT_4 showed significant inverse associations with BDE-99 [adjusted (adj) β tertile 2 vs. 1: -0.12; 95% CI: -0.24, -0.01. adj β tertile 3 vs. 1: -0.16; 95% CI: -0.28, -0.04], BDE-154 (adj β tertile 3 vs. 1: -0.14; 95% CI: -0.25, -0.02), and BDE-196 (adj β tertile 3 vs. 1: -0.13; 95% CI: -0.25, -0.003).

Single pollutant, crude models revealed a significant decrease in TT_3 with increasing BDE-47, -100, -197, -203, -207, -208, ΣPBDEs, PCB-101, -156, -169, OCDD, ΣPCDD/Fs, and ΣPOPs (Table S13). After adjustment, TT_3 showed significant negative associations with BDE-47 (adj β tertile 3 vs. 1: -0.12; 95% CI: -0.22, -0.02), BDE-99 (adj β tertile 3 vs. 1: -0.10; 95% CI: -0.21, -0.002), BDE-100 (adj β tertile 3 vs. 1: -0.12; 95% CI: -0.22, -0.02), BDE-197 (adj β tertile 3 vs. 1: -0.11; 95% CI: -0.21, -0.01), BDE-203 (adj β tertile 3 vs. 1: -0.14; 95% CI: -0.24, -0.03), BDE-

207 (adj β tertile 3 vs. 1: -0.11; 95% CI: -0.20, -0.01), and OCDD (adj β tertile 3 vs. 1: -0.10; 95% CI: -0.20, -0.003) (Fig. 1).

Single-pollutant, crude models showed a significant decrease in TrT_3 with increasing BDE-47, -99, -100, -154, -183, -203, and 1,2,3,4,6,7,8-HpCDD (Table S13). In adjusted models, TrT_3 was significantly inversely associated with BDE-47 (adj β tertile 2 vs. 1: -0.24; 95% CI: -0.44, -0.04), BDE-99 (adj β tertile 3 vs. 1: -0.27; 95% CI: -0.48, -0.06), BDE-183 (adj β tertile 3 vs. 1: -0.21; 95% CI: -0.41, -0.01), and BDE-203 (adj β tertile 3 vs. 1: -0.24; 95% CI: -0.46, -0.02) (Fig. 1).

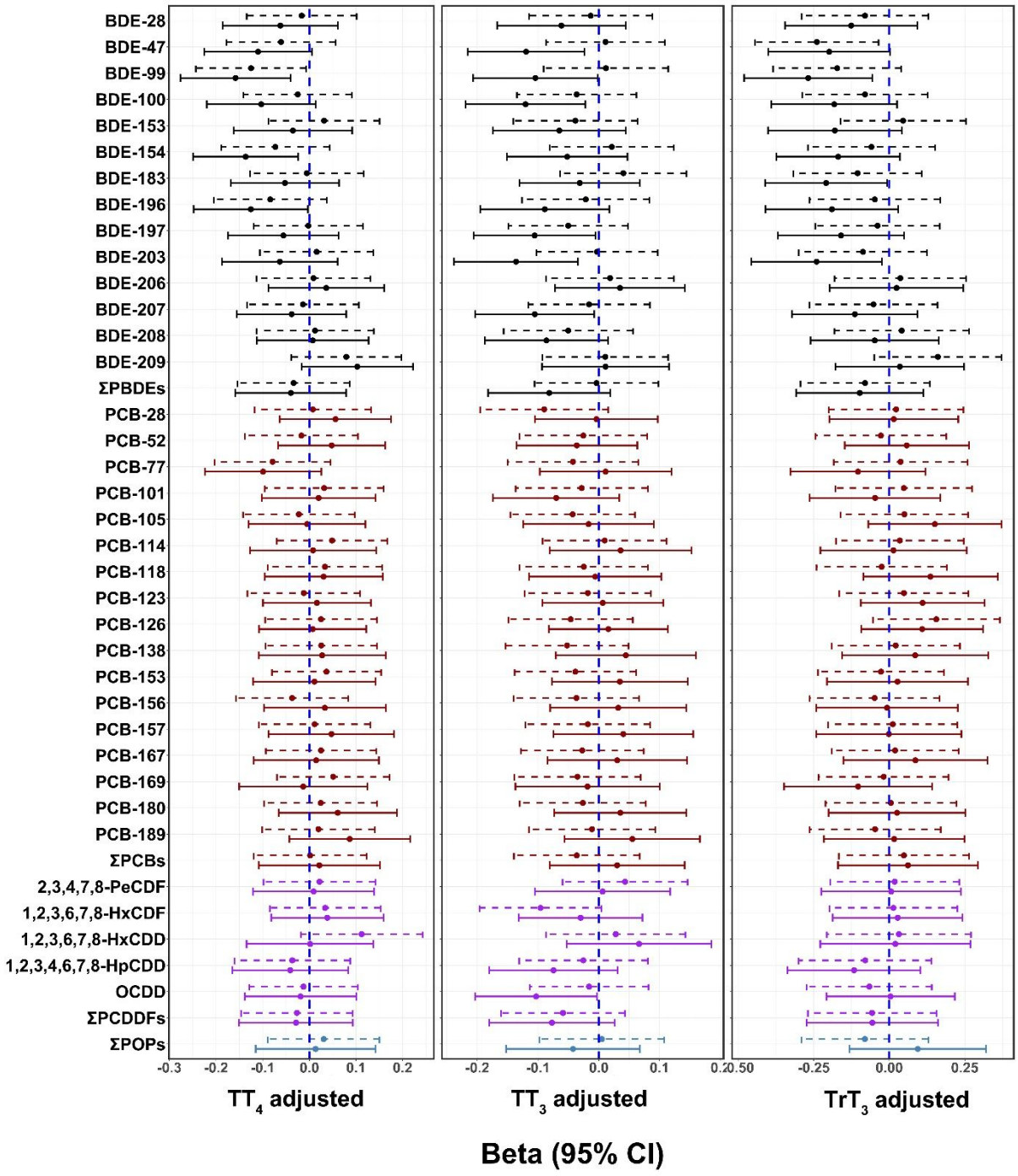

Fig. 1 Adjusted single pollutant models show the associations between exposure to tertiles of 36 POPs and THs in human breast milk. Dashed lines represent the associations of tertile 2 vs. 1 while the straight lines represent the associations of tertile 3 vs. 1. The estimated effects and corresponding confidence intervals (95% CI) are shown by dots and error bars, respectively.

3.4 Multi-pollutant model

Factor analysis

Using PCA on the 36 POPs, we generated five factors that sufficiently accounted for the total variance inherent in the data. Table S14 presents the factor loadings. As shown in Table 2, in the model that simultaneously included all five factors, exposure to tertile 3 of factor 3 (highly loaded with BDE-28, -47, -99, -100, -154, -183, and -197) was associated with significant decreases in TT_4 (adj β: -0.16; 95% CI: -0.29, -0.04) and TrT_3 (adj β: -0.29; 95% CI: -0.52, -0.06). However, TT_3 demonstrated a nonsignificant decrease (adj β: -0.10; 95% CI: -0.22, 0.01) in tertile 3 of factor 3. Similar results were observed in single-factor models, in which TT_4 (adj β: -0.12; 95% CI: -0.23, 0.00) and TrT_3 (adj β: -0.21; 95% CI: -0.41, 0.00) were significantly negatively associated with factor 3 in tertile 3, whereas nonsignificant association was found for TT_3 (adj β: -0.10; 95% CI: -0.19, 0.00). Besides, exposure to factor 4 (highly loaded with PCB-28, -105, -118, -123, -126, 1,2,3,4,6,7,8-HpCDD, OCDD, 2,3,4,7,8-PeCDF, and 1,2,3,6,7,8-HxCDF) was significantly positively associated with TT_4 (adj β: 0.13; 95% CI: 0.00, 0.26) and TrT_3 (adj β: 0.26; 95% CI: 0.03, 0.49) in tertile 2.

Table 2 Associations between exposure to tertiles of five factors from principal component analysis and TH levels based on single- and multiple-factor models.

	Single-factor model β (95% CI)			Multi-factor model β (95% CI)		
	TT_4	TT_3	TrT_3	TT_4	TT_3	TrT_3
Factor 1						
1	Reference	Reference	Reference	Reference	Reference	Reference
2	0.02 (-0.10–0.14)	0.04 (-0.07–0.14)	0.00 (-0.21–0.21)	0.02 (-0.10–0.14)	0.05 (-0.06–0.15)	-0.02 (-0.24–0.20)

3	0.05 (-0.08–0.19)	0.07 (-0.04–0.19)	0.02 (-0.22–0.26)	0.05 (-0.09–0.19)	0.08 (-0.04–0.20)	-0.02 (-0.28–0.23)
Factor 2						
1	Reference	Reference	Reference	Reference	Reference	Reference
2	0.00 (-0.12–0.12)	-0.01 (-0.12–0.09)	0.02 (-0.20–0.24)	0.03 (-0.10–0.17)	0.00 (-0.12–0.12)	0.12 (-0.12–0.37)
3	0.01 (-0.11–0.14)	-0.04 (-0.15–0.06)	-0.05 (-0.27–0.17)	0.09 (-0.04–0.22)	0.00 (-0.12–0.11)	0.08 (-0.16–0.32)
Factor 3						
1	Reference	Reference	Reference	Reference	Reference	Reference
2	-0.03 (-0.14–0.09)	-0.02 (-0.12–0.08)	-0.08 (-0.29–0.12)	-0.07 (-0.19–0.06)	-0.04 (-0.14–0.07)	-0.16 (-0.38–0.07)
3	-0.12 (-0.23–0.00)*	-0.10 (-0.19–0.00)#	-0.21 (-0.41–0.00)*	-0.16 (-0.29–-0.04)*	-0.10 (-0.22–0.01)#	-0.29 (-0.52–-0.06)*
Factor 4						
1	Reference	Reference	Reference	Reference	Reference	Reference
2	0.09 (-0.03–0.21)	0.04 (-0.06–0.15)	0.20 (-0.01–0.42)#	0.13 (0.00–0.26)*	0.06 (-0.06–0.17)	0.26 (0.03–0.49)*
3	-0.02 (-0.14–0.10)	-0.04 (-0.14–0.06)	0.10 (-0.11–0.30)	-0.01 (-0.13–0.11)	-0.03 (-0.14–0.07)	0.12 (-0.10–0.33)
Factor 5						
1	Reference	Reference	Reference	Reference	Reference	Reference
2	-0.05 (-0.17–0.07)	-0.07 (-0.18–0.03)	-0.04 (-0.26–0.17)	-0.03 (-0.15–0.09)	-0.05 (-0.16–0.06)	-0.02 (-0.24–0.21)
3	0.03 (-0.09–0.15)	0.01 (-0.10–0.11)	0.08 (-0.13–0.29)	0.01 (-0.11–0.13)	-0.01 (-0.11–0.10)	0.05 (-0.17–0.27)

All models were adjusted for maternal age, education level, parity, ethnicity, smoking, diet, and breastfeeding duration. Factor 1 loaded with PCB-114, -138, -153, -156, -157, -167, -169, -180, -189, BDE-153, and 1,2,3,6,7,8-HxCDD. Factor 2 loaded with BDE-196, -203, -206, -207, -208, and -209. Factor 3 loaded with BDE-28, -47, -99, -100, -154, -183, and -197. Factor 4 loaded with PCB-28, -105, -118, -123, -126, 1,2,3,4,6,7,8-HpCDD, OCDD, 2,3,4,7,8-PeCDF, and 1,2,3,6,7,8-HxCDF. Factor 5 loaded with PCB-52, -77, and -101.

Hierarchical clustering

241 POPs were categorized into four groups using hierarchical clustering (Table 3 & Fig. S5). Group
242 1 included PCB-114, -138, -153, -156, -157, -167, -169, -180, and -189; Group 2 included PCB-
243 28, -118, -126, 1,2,3,6,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD, 2,3,4,7,8-PeCDD, and
244 1,2,3,6,7,8-HxCDF; Group 3 included BDE-28, -47, -99, -100, -153, -154, -183, and -197; Group
245 4 included BDE-196, -203, -207, and -208. Multiple linear regression models demonstrated that
246 TT_4 (adj β: -0.13; 95% CI: -0.23, -0.02) and TrT_3 (adj β: -0.21; 95% CI: -0.42, 0.00) were
247 significantly negatively associated with Group 3 in tertile 3. Besides, TT_3 was significantly
248 inversely associated with Group 4 in tertile 3 (adj β: -0.12; 95% CI: -0.23, -0.01).
249 **Table 3** Associations between POPs and THs. POPs were categorized based on hierarchical
250 clustering.

	TT_4 β (95% CI)	TT_3 β (95% CI)	TrT_3 β (95% CI)
Group 1			
1	Reference	Reference	Reference
2	0.01 (-0.12–0.13)	-0.02 (-0.13–0.08)	-0.04 (-0.25–0.18)
3	0.07 (-0.08–0.22)	0.08 (-0.04–0.21)	-0.02 (-0.28–0.24)
Group 2			
1	Reference	Reference	Reference
2	0.04 (-0.09–0.17)	-0.02 (-0.13–0.09)	-0.04 (-0.26–0.18)
3	0.05 (-0.10–0.19)	0.02 (-0.10–0.15)	0.17 (-0.08–0.42)
Group 3			
1	Reference	Reference	Reference
2	-0.04 (-0.16–0.08)	-0.02 (-0.12–0.09)	-0.06 (-0.27–0.15)
3	-0.13 (-0.23–-0.02)*	-0.06 (-0.17–0.04)	-0.21 (-0.42–0.00)*
Group 4			
1	Reference	Reference	Reference

2	0.01 (-0.12–0.14)	-0.02 (-0.12–0.09)	-0.01 (-0.23–0.22)
3	-0.05 (-0.18–0.09)	-0.12 (-0.23–-0.01)*	-0.16 (-0.39–0.07)

All models were adjusted for maternal age, education level, parity, ethnicity, smoking, diet, and breastfeeding duration. Group 1 included PCB-114, -138, -153, -156, -157, -167, -169, -180, and -189. Group 2 included PCB-28, -118, -126, 1,2,3,6,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD, 2,3,4,7,8-PeCDD, and 1,2,3,6,7,8-HxCDF. Group 3 included BDE-28, 47, -99, -100, -153, -154, -183, and -197. Group 4 included BDE-196, -203, -207, and -208.

3.5 Sensitivity analysis

Similar results were obtained when POPs were modeled in ng/g lw or in ng/L milk. The models kept robust when BMI was further controlled. As shown in Table S15, in multifactor model, exposure to tertile 3 of factor 3 was significantly inversely associated with TT_4 (adj β: -0.16; 95% CI: -0.29, -0.03) and TrT_3 (adj β: -0.30; 95% CI: -0.53, -0.07). Similar results were observed in single-factor models, in which TT_4 (adj β: -0.12; 95% CI: -0.24, 0.00) and TrT_3 (adj β: -0.21; 95% CI: -0.41, 0.00) showed significant negative associations with factor 3 in tertile 3. Exposure to factor 4 was significantly positively associated with TT_4 (adj β: 0.15; 95% CI: 0.01, 0.29) and TrT_3 (adj β: 0.26; 95% CI: 0.02, 0.51) in tertile 2.

4. Discussion

THs were quantified in human breast milk using LC-MS/MS for the first time. TT_4 and TT_3 levels measured here were similar to a previous report measured in preterm breast milk using radioimmunoassay (RIA), while higher TT_4 was found in term breast milk (see Table S3) [25]. This is probably due to the differences in the time of sampling. However, our results might be more reliable and accurate since IA technology is prone to nonspecific interferences. Additionally, due to their low concentrations, THs in human breast milk is not an adequate source for infants with congenital hypothyroidism [25].

Compared with BAMBI data from 2007–2008, this cohort had substantially lower exposure to POPs, with PCDD/Fs and dl-PCBs decreased for 52% and 44%, respectively [30]. Besides, the POP

levels were generally lower compared with values reported in other regions. For example, PBDE and PCB levels measured here were lower than those reported in the North America [3], while PCDD/Fs were lower than those measured in India [40]. Therefore, our study represents a low-exposure population.

4.1 Associations of PBDEs with THs

We observed the highest thyroid-disrupting potencies for PBDEs among the POPs examined. Single pollutant models revealed significant inverse associations of THs with BDE-47, -99, -100, -154, -183, -196, -197, -203, and -207. Similar and robust results were obtained in multipollutant models using PCA and hierarchical clustering, which proved a significant association of increasing PBDEs (including BDE-28, -47, -99, -100, -154, -183, and -197) with depressed TT_4 and TrT_3. These findings are consistent with previous epidemiologic studies [13, 20, 41]. Animals studies using rats [42], fish [43], kestrels [44], and minks [45] also proved decreased THs following PBDE exposure. Putative mechanisms include the interference of PBDEs with TH transport and metabolism. For example, *in vitro* studies demonstrated that lower-brominated OH-PBDEs are structurally similar to THs and can competitively bind with TR [46, 47]. Enhanced TH metabolism, in combination with elevated cytochrome P450 enzymes 2B (CYP2B) expression, deiodinase I (DIO1) enzyme activity, as well as the gene expression of Cyp2b1/2, dio1, and hepatic efflux transporters were observed in rats following DE-71 (predominately composed of BDE-47, 99, -100, -153, and -154) exposure [48]. In addition, PBDEs may disrupt the HPT axis by interfering with the TSHβ expression [43].

In contrast, others observed positive or nonsignificant associations, and the associations might be differed by sex [15, 17, 49]. The inconsistence is probably the results of random error given the intraindividual and inter-individual variability in TH set-points. For example, Stapleton *et* al. [15]

298 and Zota *et* al. [49] employed serum samples from women during pregnancy when marked
299 fluctuations in HPT axis homeostasis occur [50]. Our samples were collected post-partum when HPT
300 axis tends to be more stable [50].

301 Another possibility is that the relationship between PBDEs and THs may vary by exposure level.
302 THs act at quite low concentrations (free serum T_4 (FT_4) level: 8–20 ng/L [51]) while low-dose
303 effects and non-monotonic responses are remarkably common in studies of EDC (low-dose cutoff
304 of BDE-99: 0.3 mg/kg d) [22]. Abdelouahab *et* al. observed significant decreases in TT_4 and TT_3 in
305 lambs following low-dose exposure of BDE-47 [52]. A meta-analysis suggested that the relationship
306 between THs and PBDEs might be an inverted U-shape curve [19]. Inverse association was found in
307 populations with low- [23, 41] and high-dose PBDE exposures [20], while positive association was
308 reported in a population exposed with middle-level PBDEs [53]. This is generally in agreement with
309 our findings because of the low exposure levels of POPs in this study. Our results proved negative
310 associations of low level PBDE exposure with THs. However, caution should be taken when
311 comparing our results to previous findings, given variation in study design, population
312 susceptibility, exposure level, biomarker measurement, as well as sample type which contain
313 different contents of lipid, TH-binding proteins, and enzymes. Further studies are warranted to
314 clarify the underlying mechanisms.

315 **4.2 Associations of PCBs with THs**

316 Dl-PCBs and dioxins may upregulate UDPGA by binding with aryl hydrocarbon receptor
317 (AhR), leading to enhanced excretion of T_4. Non-dl-PCBs, however, may interfere with the
318 activities of CYP enzymes (i.e., CYP 2B1 and CYP 3A1) which may also reduce circulating T_4 [54,
319 55]. A substantial body of animal and epidemiologic studies have reported decreased THs with
320 increasing exposure of PCBs, despite the literature is inconsistent [18, 56-58]. Langer *et* al. reported

negative associations of FT_4 and TT_3 with PCBs at low exposure levels (serum level: < 530 ng/g lw), but positive relations at higher levels [59]. A recent study also observed inverse associations between low-dose PCBs and TT_3, Free T_3, and FT_4 in a Chinese population [8]. These findings are in line with our results of single-pollutant crude models, which revealed significant inverse associations of PCB-101, -156, and -169 with THs, although they were insignificant after adjustment. In contrast, multipollutant analysis revealed significant positive associations of TT_4 and TrT_3 with Factor 4, which was mainly loaded with PCBs and PCDD/Fs. This probably indicates that the relationship between PCBs and THs can be influenced by PCDD/Fs. Similarly, in a population highly exposed with polybrominated biphenyls (PBBs) from Michigan, serum PCBs were found to be positively associated with THs [36].

4.3 Associations of PCDD/Fs and PBDD/Fs with THs

1,2,3,6,7,8-HxCDD, OCDD, and 1,2,3,4,6,7,8-HpCDD showed significant inverse associations with TT_4 and TT_3 in crude single-pollutant models. After adjustment, only the association of OCDD with TT_3 remained. Our previous study observed that placental TT_4 was significantly inversely associated with 2,3,7,8-TCDD, but significantly positively associated with 1,2,3,6,7,8-HpCDF, TT_3 was significantly positively associated with 2,3,7,8-TCDF and 1,2,3,7,8-PeCDF, while TrT_3 was significantly positively associated with 1,2,3,7,8-PeCDF and 2,3,4,6,7,8-HxCDF [18]. Other studies also reported inconsistent results [60, 61]. The probable reasons include the presence of different pollutant mixtures, varying timing of sampling, exposure level, and uncontrolled bias.

PBDD/Fs are brominated dioxins found as impurities of PBDEs and formed during the incineration and degradation of brominated chemicals [62]. Similar to previous reports [63, 64], we observed low DFs for PBDD/Fs. Therefore, the thyroid-disrupting properties of these chemicals were not assessed here.

4.4 Strengths and limitations

Our study has several strengths: (1) THs in human breast milk was measured for the first time to investigate the thyroid-disrupting effects of POPs. Compared with serum, we obtained much higher detection frequencies for POPs. This is because it is easier to obtain large sample amount, as well as the high lipid content in milk; (2) A wide variety of POPs and potential confounders were measured and included in the statistical analysis, which can provide an overview of possible relationships between POPs and THs. Furthermore, multipollutant approaches enabled us to evaluate the integrated effects of POP mixtures. This is critical because many POP congeners show similar chemical and biological properties; (3) The low exposure of POPs in this population enabled us to estimate the thyroid-disrupting effects of POPs in background low-exposure population. Our study also has certain limitations. For example, we did not measure serum TH levels of infants that are more susceptible to thyroid disruption. Besides, with human breast milk we can only assess the maternal TH homeostasis, and therefore we are not able to estimate the sex-specific associations between POPs and THs. Additional limitations include the lack of thyroid-binding protein levels and the OH-PBDEs and OH-PCBs, which in general show higher potencies of thyroid-disruption. Lastly, this study was limited by the small sample size which may reduce the statistical power.

ASSOCIATED CONTENT

Supporting Information

The following files are available free of charge.

Analytical methods of THs in human breast milk, comparison of POPs in different populations, correlations between biomarkers, crude single-pollutant models, factor loadings, and directed acyclic graph (PDF).

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGEMENTS

Z.-M. Li was supported by the China Scholarship Council (No. 201604910584). The authors would like to thank Agilent Technologies for supporting this research project.

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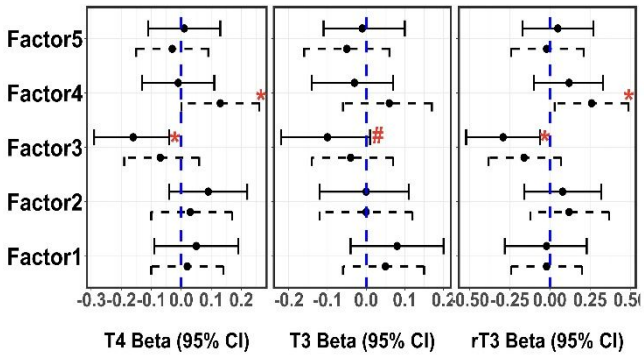
590 **Table of Contents (TOC) Graphic**



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