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Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Thyroid Hormone Determination and Associations with Environmental Exposure and Gestational Diabetes Mellitus

Zhongmin Li

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Abstract

Exposures to endocrine-disrupting chemicals (EDCs) have been implicated in the etiologies of a wide array of adverse outcomes. The work represented herein focused on the human exposomics of persistent organic pollutants (POPs) in relation to endogenous hormone biomarkers, namely thyroid hormones (THs) and monoamines. THs play critical roles in the protein, carbohydrate, and lipid metabolism, neural development, heart rate, as well as brain, renal, and cardiovascular functions. For this purpose UPLC-Q-TOF-MS and HPLC-QqQ-MS approaches in combination with isotope dilution technology were developed for the determination of THs, viz. 3,5,3',5'-L-thyroxine (T_4), 3,3',5-triiodo-L-thyronine (T_3), 3,3',5'-triiodo-L-thyronine (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), in human placenta and breast milk. The methods were evaluated with linearity range, limits of detection and quantification, spike-recoveries, matrix effects, and intra-day and inter-day variations. The optimized methods showed high sensitivity and selectivity. Besides, HPLC-QqQ-MS technology was employed to quantify endogenous 3-T_1AM in rodent serum and tissues. The results proved that $^{13}C_6$-T_1AM is a proper quantification standard for 3-T_1AM measurement. Using this technology, 3-T_1AM was quantified in the liver of mouse following administration with NAc-T_1AM and OAc-T_1AM.

The thyroid-disrupting effects of POPs were investigated using human placenta and human breast milk samples collected from the Danish EXPORED cohort (1991-2001, Copenhagen, Denmark) and German LUPE cohort (2015-2016, Bavaria, Germany), respectively. THs and POPs including polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), polybrominated dibenzo-*p*-dioxins and furans (PBDD/Fs), organochlorine pesticides (OCPs), and organotin compounds (OTCs) were measured. Several models were applied to estimate the relationship of THs with POPs including multiple linear regression, principal component analysis, partial least squares regression, and hierarchical clustering. The results illustrated associations between THs and certain POPs.

The associations between placental THs and the odds of gestational diabetes mellitus

(GDM) was evaluated in a Chinese birth cohort. Placenta samples from women with (32) and without (85) GDM were collected. The association between GDM and THs were analyzed using multiple logistic regression. The results revealed no significant associations. However, due to the small sample size (32) in this study, the statistical power might be limited. Further studies are warranted.

Reliability in the use of placental biomarkers requires an understanding of their distributions. The placental distribution of THs (T_4, T_3, and rT_3), elements (As, Cd, Cr, Hg, Pb, Se, Zn, Mn, Cu, Fe, Ca, K, Mg, Na, and Al), POPs (PBDEs, PCBs, and PCDD/Fs), and monoamines, were examined in human and porcine placentomes. Different distribution properties for different chemicals were observed. Heterogeneous distributions were found for certain THs, elements, and PCDD/Fs, while PCBs and PBDEs showed high reliability. The results suggested different modes of placental distribution and transfer, and highlighted the challenges of assessing intrauterine exposures because of the sampling bias. Different sampling strategies should be applied according to their distribution variations.

Finally, a sample pooling approach was applied to investigate the relationship between type 2 diabetes (T2D) and POP exposures in the German CARLA ("Cardiovascular Disease-Living and Aging in Halle", 2002-2006, East Germany) cohort. Significant differences were observed in the levels of PCB-105, -114, -118, -138, -153, -156, -157, -167, -180, -189, β-HCH, HCB, octachlorostyrene, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, oxychlordane, and cis-heptachlor epoxide between pooled cases and pooled controls. These results proved that sample pooling is a useful explorative tool in Exposome Wide Association Studies (EWAS).

Overall, the optimized analytical methods were proved to be highly sensitive and selective. Certain POPs showed significant associations with TH biomarkers. Considering the critical role of THs in physiological processes, the results in this dissertation are of great public health significance.

Zusammenfassung

Die Exposition gegenüber endokrin wirkenden Chemikalien (EDCs) ist mit einer Vielzahl von Nebenwirkungen verbunden. Die hier vorgestellten Arbeiten konzentrierten sich auf das Exposom persistenter organischer Schadstoffe (POPs) in Bezug auf Schilddrüsenhormone (THs) als endogene Biomarker. Zu diesem Zweck wurden UPLC-Q-TOF-MS- und HPLC-QqQ-MS-Methoden in Kombination mit Isotopenverdünnungstechnologie zur Bestimmung von THs entwickelt, im Einzelnen 3,5,3',5'-$_L$-thyroxin (T_4), 3,3',5-triiod-$_L$-thyronin (T_3), 3,3',5'-triiod-$_L$-thyronin (rT_3), 3,3'-diiod-$_L$-thyronin (3,3'-T_2), 3,5-diiod-$_L$-thyronin (3,5-T_2), 3-iod-$_L$-thyronin (T_1) und 3-iodthyronamin (3-T_1AM) in menschlicher Plazenta und Muttermilch. Die Methoden wurden mit Linearitätsbereich, Nachweis- und Bestimmungsgrenzen, Spike-Recovery, Matrixeffekten, tages- und tagesübergreifenden Schwankungen bewertet. Außerdem wurde die HPLC-QqQ-MS-Technologie eingesetzt, um endogenes T_1AM in Nagetierserum und -geweben zu quantifizieren. Obwohl begrenzte Fortschritte erzielt wurden, erwies sich nur $^{13}C_6$-T_1AM als geeigneter Quantifizierungsstandard für 3-T_1AM.

Es wurden Wirkungen von POPs unter Verwendung von menschlicher Plazenta und menschlicher Muttermilch, die im Rahmen des dänischen EXPORED-Projekts bzw. des deutschen LUPE-Projekts gesammelt wurden, auf die Schilddrüsenhormonhomöostase untersucht. THs und polybromierte Diphenylether (PBDEs), polychlorierte Biphenyle (PCBs), polychlorierte Dibenzo-p-dioxine und Furane (PCDD/Fs), polybromierte Dibenzo-p-dioxine und Furane (PBDD/Fs) und Organozinnverbindungen (OTCs)) wurden gemessen. Um die Beziehung von THs zu POPs abzuschätzen wurde multiple lineare Regression, Hauptkomponentenanalyse, partielle Regression kleinster Quadrate und hierarchische Clusterbildung auf die Datensätze angewendet. Unsere Ergebnisse zeigten Assoziationen zwischen THs und bestimmten POPs.

Assoziationen zwischen plazentalen THs und Schwangerschaftsdiabetes (GDM) in einer chinesischen Geburtskohorte wurde mittels multipler logistischer Regression analysiert. Die Ergebnisse zeigten keine signifikanten Assoziationen zwischen Plazenta-THs und GDM. Die Zuverlässigkeit der Verwendung von Plazenta-Biomarkern setzt deren Verständnis ihrer Verteilung in der Plazenta voraus. Die Plazentaverteilung und der Plazentatransfer von THs (T_4,

T$_3$ und rT$_3$) und Elementen (As, Cd, Cr, Hg, Pb, Se, Zn, Mn, Cu, Fe, Ca, K, Mg, Na und Al)), POPs (PBDEs, PCBs und PCDD/Fs) und Monoaminen in Plazentomen von Menschen und Schweinen wurde dahigehend charakterisiert. Heterogenität ergab sich für Verteilungen von THs, Elementen und PCDD/Fs, während PCBs und PBDEs eine hohe Homogenität aufwiesen. Unsere Ergebnisse legten verschiedene Arten der Plazentaverteilung und -übertragung nahe und hoben die Herausforderungen bei der Beurteilung der intrauterinen Exposition aufgrund der Stichprobenverzerrung hervor. Verschiedene Probenahmestrategien sollten entsprechend ihrer Verteilungsvariation angewendet werden.

Verwendung eines Sample-Pooling-Ansatz, um den Zusammenhang zwischen Typ-2-Diabetes (T2D) und POP-Expositionen in der deutschen CARLA-Kohorte zu untersuchen. ergab signifikante Unterschiede in den Konzentrationen von PCB-105, -114, -118, -138, -153, -156, -157, -167, -180, -189, β-HCH, HCB, Octachlorstyrol, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, Oxychlordan und cis-Heptachlor-Epoxid zwischen gepoolten Fällen und gepoolten Kontrollen. Diese Ergebnisse haben gezeigt, dass das Sammeln von Stichproben ein nützliches exploratives Werkzeug in Exposome Wide Association Studies (EWAS) ist.

Insgesamt erwiesen sich die optimierten Analysemethoden als hochsensibel und selektiv. Bestimmte POPs zeigten signifikante Assoziationen mit TH-Biomarkern. Angesichts der entscheidenden Rolle von THs für physiologische Prozesse sind die Ergebnisse von großer Bedeutung für die öffentliche Gesundheit.

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List of abbreviations and acronyms

TH	Thyroid hormone
T_4	3,5,3',5'-L-Thyroxine
T_3	3,5,3'-L-triodothyronine
rT_3	3,3',5'-L-triiodothyronine
$3,3'-T_2$	3,3'-diiodo-L-thyronine
$3,5-T_2$	3,5-diiodo-L-thyronine
T_1	Monoiodothyronine
$3-T_1AM$	3-iodothyronamine
T_0AM	Thyronamine
HPT	Hypothalamus-pituitary-thyroid
POP	Persistent organic pollutant
PBDE	Polybrominated diphenyl ether
BFR	Brominated flame retardant
PCB	Polychlorinated biphenyl
OCP	Organochlorinated pesticide
PCDD/F	Polychlorinated dibenzo-*p*-dioxin and furan
PBDD/F	Polybrominated dibenzo-*p*-dioxin and furan
OTC	Organotin compound
GDM	Gestational diabetes mellitus
T2D	Type 2 diabetes
T1D	Type 1 diabetes
UPLC-Q-TOF-MS	Ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry
HPLC-QqQ-MS	High performance liquid chromatography triple quadrupole mass spectrometry
HRGC-HRMS	High resolution gas chromatography- high resolution mass spectrometry
ICP-MS	Inductively coupled plasma mass spectrometry

PCA   Principal component analysis

PLS   Partial least squares

DAG   Directed acyclic graph

EWAS   Exposome Wide Association Studies

Chapter 1 Introduction

1.1 Thyroid hormones

Thyroid hormones (THs) are a class of tyrosine-based hormones indispensable for the proper physiological state of the body. THs are produced from the thyroid gland, which mainly includes $_L$-3,5,3',5'-thyroxine (T_4) and a smaller fraction of biologically active $_L$-3,5,3'-triodothyronine (T_3) (T_4/T_3 ratio of 4:1 to 5:1) (shown in Fig. 1.1). T_4 is synthesized in the thyroid follicular cells in thyroid gland in the presence of thyroglobulin, thyroid peroxidase, and iodide. Generally, five steps are involved in this process (1) I^{-1} is transferred to the thyroid follicular cells from plasma through the sodium iodide symporter (NIS); (2) formation of H_2O_2 through peroxidase; (3) iodination of tyrosyl residues of thyroglobulin by thyroid peroxidase and H_2O_2; (4) thyroid peroxidase catalyzes the phenolic coupling of iodotyrosyl residues of thyroglobulin to generate T_4; (5) release of T_4 from thyroglobulin [1].

The hypothalamus-pituitary-thyroid (HPT) axis composed of hypothalamus, pituitary gland, and thyroid gland controls the TH homeostasis [2]. Thyrotropin-releasing hormone (TRH) produced from the neurons in the hypothalamus stimulates the pituitary gland to secrete thyrotropin/thyroid-stimulating hormone (TSH). The binding of TSH with thyroid via a G protein-coupled receptor (GPCR) stimulates the production of T_4 and T_3, which are released into the blood. The level of TSH is regulated by T_4 and T_3 in brain and hypothalamus through negative feedback.

T_4 is transported to target organs and cells by a variety of transfer proteins including thyroxine-binding globulin (TBG), transthyretin (TTR), serum albumin, monocarboxylate transporter 8 (MCT8) and MCT10 [1]. Peripheral deiodinations are controlled by iodothyronine deiodinases (Dios), which are differentially and developmentally expressed in tissues. The expression of type 1 iodothyronine deiodinases (Dio1) is primarily in the thyroid, liver and kidney; type 2 iodothyronine deiodinases (Dio2) in the thyroid, brain, pituitary, and brown adipose tissue (BAT); and type 3 iodothyronine deiodinases (Dio3) in the placenta, vascular tissue and skin. Dio1 catalyzes deiodinations of both the phenolic ring and tyrosyl ring. Dio2 only exhibit phenolic ring deiodination activity, while Dio3 only catalyzes deiodination at tyrosyl ring. Monodeiodination at phenolic ring of T_4 by Dio1 and Dio2 produces T_3 [3]. In contrast, tyrosyl-ring deiodination by Dio1 and Dio3 inactivates TH by converting T_4 into 3,3',5'-$_L$-triiodothyronine (reverse T_3 or rT_3). Dio1 can also convert both T_3

and rT_3 into $3,3'$-T_2 or $3,5$-T_2 [4]. $3,3'$-T_2 and $3,5$-T_2, in turn, can give rise to monoiodothyronine (T_1) and iodine-free thyronine (T_0) through further deiodination events. Decarboxylation yields decarboxylated metabolites thyronamines (TAMs), i.e., 3-iodothyronamine (3-T_1AM) [5, 6]. Conjugation of phenolic hydroxyl groups with sulfate or glucuronic acid increases water solubility of substrates, facilitating biliary and/or urinary clearance. In addition, deamination of THs leads to the formation of the so-called acetic acid-TH analogs such as $3,3',5$-triiodothyroacetic (Triac) and $3,3',5,5'$-tetraiodothyroacetic (Tetrac) acids [5]. The molecular structures of THs and metabolites are shown in Fig. 1.1.
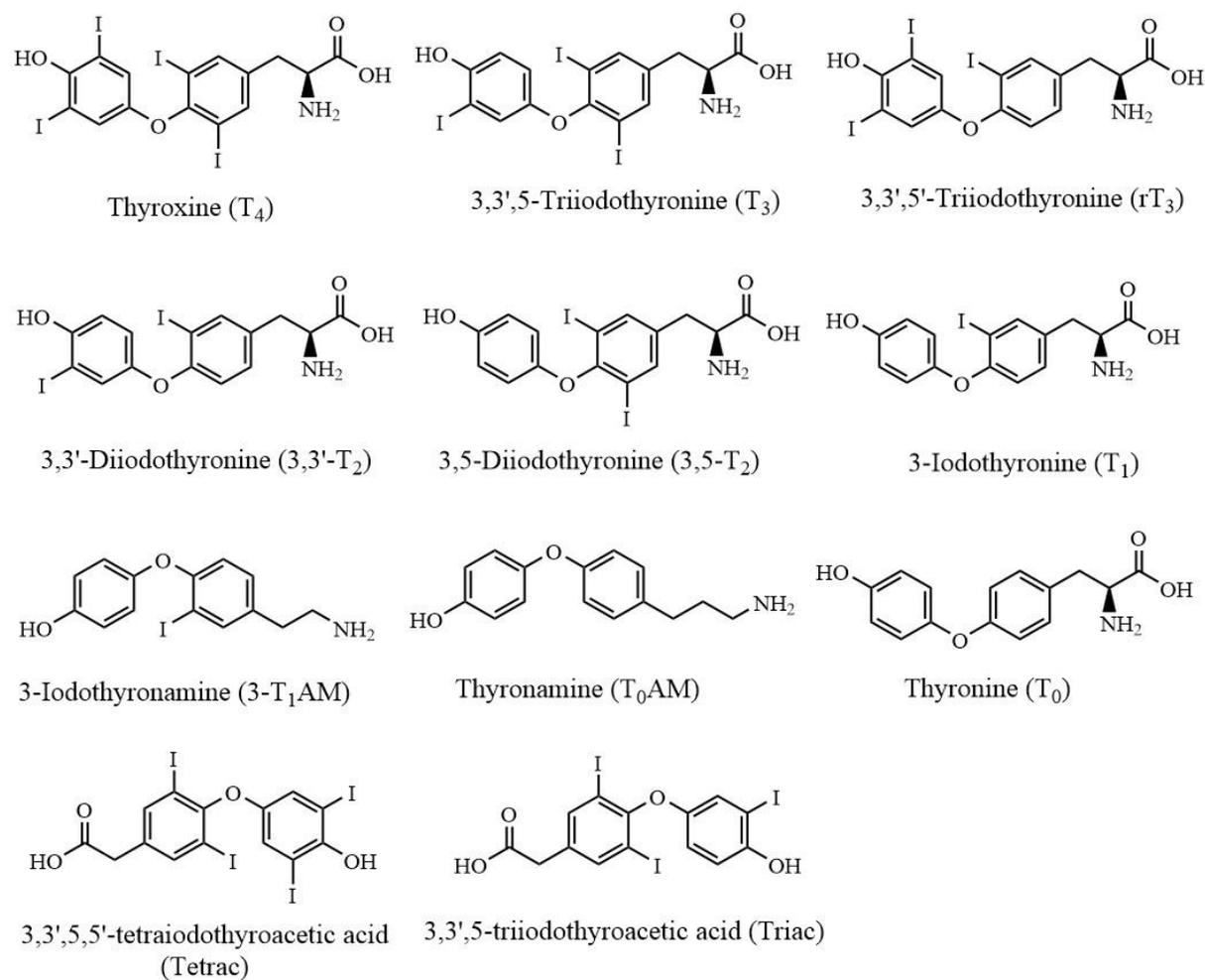
Fig. 1.1 Chemical structures of thyroid hormones and metabolites.

THs regulate a bulk of physiological processes and are important biomarkers. T_4 and T_3 are responsible for the regulation of oxygen consumption, carbohydrate metabolism, protein synthesis, and fetal neurodevelopment [7]. The function of T_4 and T_3 is primarily exerted via thyroid hormone receptor (TR), which belongs to the nuclear receptor superfamily and possesses the common protein domain structure. The TR structure is mainly composed of a

zinc finger motif DNA binding domain and a COOH-terminal domain that mediates ligand interactions and binding of coactivators and corepressors [3]. TRα and TRβ are two primary isoforms of TR that differently expressed in most vertebrates. TRα is dominant in the brain and skeletal system while TRβ is abundant in liver. T_3 shows higher affinity in binding with TR than T_4. TRs belong to type II receptors, which are retained in the nucleus regardless of the ligand binding status and in addition bind as hetero-dimers. In the absence of hormones, TRα and TRβ dimerize with a retinoid X receptor (RXR). The dimer binds to the hormone response elements (HRE) of the target gene together with co-repressor proteins. Binding of THs leads to a conformational change in the TR which then releases co-repressors and recruits co-activator proteins that are responsible for the transcription of target genes [8-10].

Nongenomic actions of THs are initiated at the plasma membrane receptor on integrin avβ3 or in cytoplasm. Via the integrin receptor, TH from the cell surface stimulates MAPK (ERK1/2) through phospholipase C (PLC) and protein kinase C (PKC). In cytoplasm, T_3 can nongenomically activate PI3K and initiate downstream transcription of specific genes [11]. Activation of PI3K can involve TRβ1 or TRα resident in cytoplasm. A truncated form of TRα1 (TRΔα1) in cytoplasm mediates the action of T_4 and rT_3 on the action cytoskeleton. T_3 and T_4 may also activate PI3K from the integrin αvβ3 hormone receptor site [12].

Recent studies revealed important novel properties of other TH congeners. For example, rT_3 is an inhibitor of T_3 activity. Thus, the T_3/rT_3 ratio is used as a clinical diagnostic marker of peripheral TH metabolism [13, 14]. $3,3'-T_2$ and $3,5-T_2$ have different activities in different tissues, including suppression of TSH levels and increase in resting metabolic rate [15, 16]. Besides, administration of $3,5-T_2$ in rats was found to reduce body weight, serum cholesterol and triglyceride, and liver fatty acid oxidation rate [17].

TAMs are suggested to be the decarboxylated metabolites of T_4 and T_3. In 2004, Scanlan *et* al. firstly reported the existence of endogenous $3-T_1AM$ in rodent and its identical biological properties. This finding boosted the research on this compound tremendously [18]. $3-T_1AM$ has been proposed to be a novel chemical messenger because of the following properties: (1) $3-T_1AM$ exist in the brain of rodent [18]; (2) $3-T_1AM$ is a multitarget ligand and can interact with the trace amino associated receptor 1 (TAAR1), certain aminergic receptors, mitochondrial proteins, apolipoprotein B-100 (apoB-100), and transient receptor potential channels [19]; (3) significant hypothermia and bradycardia were observed in mouse following administration of $3-T_1AM$; and (4) $3-T_1AM$ can reduce contractile performance and heart rate

in the isolated perfused rat heart [20].

The mechanisms of the biosynthesis of $3-T_1AM$ have not been identified unequivocally. Three pathways have been proposed: (1) sequential deiodination and decarboxylation of TH precursors. T_3AM and $3,5-T_2AM$ are possible precursors; (2) synthesis and secretion in thyroidal gland; (3) synthesis by microbiota in gut [19].

1.2 Thyroid hormone determination

Routine clinical assessment of THs has long been achieved by the measurement of T_4 and T_3 in blood by immunoassay (IA) methods [21]. The radioimmunoassay method is based on the competitive binding of plasma TH and the ^{125}I-labelled TH to a limited number of binding sites on TH antibody. The proportion of the ^{125}I-labelled TH, bound to the antibody, is inversely related to the concentration of TH present. By measuring the proportion of ^{125}I-labelled TH bound in the presence of plasma containing various known amount of THs, the concentration of THs in the unknown samples can be determined. Commercialized kits based on competitive enzyme-linked immunosorbent assay (ELISA) have been available for the quantification of total and free levels of T_4 and T_3 in serum, plasma, urine, extracted dried fecal samples, and tissue culture media samples.

IA methods provides high sensitivity, which, however, is prone to nonspecific interferences because of the limited specificity of the antibodies [22-24]. Wide interlaboratory assay variations for T_4 and T_3 results have been observed by the College of American Pathologies Proficiency Testing (CAPPT) program, depending on the antibodies used [25]. Besides, there are marked changes in the maternal HPT axis during pregnancy, resulting a 2- to 3- fold increase in thyroid hormone-binding proteins. IA methods in samples from pregnant women can give false diagnosis of TH levels [25].

Methods based on tandem mass spectrometry offer a better specificity and accuracy [26], and have been used in various samples such as human and animal serum/plasma and tissues [24, 27-34]. Table 1.1 shows the sample types, extraction methods, analytical equipment, analytes, calibration ranges and the limits of detection (LODs) or limits of quantification (LOQs) of the methods developed in recent years. The LC-MS and LC-MS/MS technologies in combination with solid-phase extraction (SPE) and isotope dilution provide sufficient sensitivity and selectivity for TH quantification in various matrices.

Table 1.1 Extraction methods, analytical equipment, analytes, calibration ranges and the limits of detection (LODs) or limits of quantification (LOQs) of the LC-MS and LC-MS/MS approaches reported recently.

Sample	Extraction and clean-up	Equipment	Analytes	Internal standards	Calibration range	LODs/LOQs	Ref.
Thyroid gland from male SD-rats	Pronase digestion, ethanol extraction	HPLC-MS/MS	T_4, T_3, rT_3, $3,3'$-T_2, $3,5$-T_2, 3-T_1		1–200 µg/L	LOQs: 0.25–0.52 µg/L	[34]
Human serum	Acetone extraction	HPLC-MS/MS	T_4, T_3, rT_3, $3,3'$-T_2, $3,5$-T_2,	$^{13}C_6$-T_4	1–500 µg/L	LODs: 1.5–7 pg on-column	[28]
Brain, thyroid gland from SD-rats	Pronase digestion, Acetone extraction	HPLC-MS/MS	T_4, T_3, rT_3, $3,3'$-T_2, $3,5$-T_2, 3-T_1	$^{13}C_6$-T_4	0.5–200 µg/L	LODs:7.5–13.5 pg on-column,13.5–16.5 pg on-column	[29]
Liver, heart, hypothalamus from male Wistar rats	Liquid-liquid extraction, SPE	UPLC-MS/MS	T_4, T_3, rT_3, $3,3'$-T_2, $3,5$-T_2,	$^{13}C_6$-T_4, $^{13}C_6$-T_3, $^{13}C_6$-rT_3, $^{13}C_6$-$3,3'$-T_2	0.23–90 nmol/L	LOQs:0.98–1.73 pg on-column	[30]
Heart from Wistar rats	Derivatization, solid-phase extraction	HPLC-MS/MS	T_4, T_3	$^{13}C_6$-T_4, $^{13}C_6$-T_3	0.2–50 µg/L	LODs: 10 ng/L	[35]
Liver, heart, kidney, muscle, lung, and BAT from C57BL/6J mouse	Solid-liquid extraction, Liquid-liquid extraction, SPE extraction	UPLC-Q-TOF-MS	T_4, T_3, rT_3, $3,3'$-T_2, $3,5$-T_2, T_1	$^{13}C_6$-T_4, $^{13}C_6$-T_3, $^{13}C_6$-rT_3, $^{13}C_6$-$3,3'$-T_2	0–100 µg/L	LOQs: 0.5–1 µg/L (2.5–5 pg on-column)	[36]

Matrix	Extraction	Instrument	Analytes	Internal standards	Calibration range	Limits	Ref
Human and mouse placenta	Solid-liquid extraction, Liquid-liquid extraction, SPE extraction	UPLC-Q-TOF-MS	T_4, T_3, rT_3, 3,3'-T_2, 3,5-T_2, T_1, 3-T_1AM	$^{13}C_6$-T_4, $^{13}C_6$-T_3, $^{13}C_6$-rT_3, $^{13}C_6$-3,3'-T_2	0–100 µg/L	MLOD: 0.01–0.2 ng/g MLOQ: 0.04–0.7 ng/g	[37]
Human serum	Liquid-liquid extraction, SPE	HPLC-Q-TOF-MS	T_4, T_3, rT_3	$^{13}C_6$-T_4		LOD[a]: 0.4 µg/L LOQ[a]: 1.0–1.4 µg/L	[38]
Frog (*Xenopus laevis*) plasma and tadpole (*Rana (Lithobates) catesbeiana*) serum)	Liquid-liquid extraction, SPE	HPLC-MS/MS	T_4, T_3, rT_3, 3,3'-T_2, 3,5-T_2, T_1, T_0, T_1AM, Diac, Triac, and Tetrac	$^{13}C_6$-T_4, $^{13}C_6$-T_3, $^{13}C_6$-rT_3, $^{13}C_6$-3,3'-T_2		MLOD: 0.01–0.38 nM MLOQ: 0.03–1.14 nM	[32]
Bovine serum	Liquid-liquid extraction, 30 kDA ultracentrifugation, SPE	UPLC-MS/MS	T_4, T_3, rT_3	$^{13}C_6$-T_4		Total THs LOD: 0.01–0.02 µg/L Total THs LOQ: 0.03–0.05 µg/L Free THs LOD: 0.9–2.2 µg/L Free THs LOQ: 2.6–6.6 µg/L	[39]

Abbreviations: MLOD, method limit of detection; MLOQ, method limit of quantification.
[a]matrix based LOD and LOQ

Although great development has been obtained in this technology, knowledge gaps still exist. The major challenges in TH analysis include developing a rapid, efficient, and reproducible sample cleanup procedure to extract THs form the sample matrix, as well as an efficient chromatographic separation, and quantification method. More than 99% of THs are bound with proteins such as thyroid-binding globulin, apoB-100, etc. Different preanalytical approaches have been employed. Organic solvent (i.e., acetonitrile, methanol alcohol) has been used to deproteinize and release T_4 and T_3, which, however, may not be sufficient [37]. Kunisue *et* al. applied a pronase digestion procedure before extraction, which was proved to be efficient to dissociate T_4 and T_3 from proteins [29]. Other attempts include deproteinization with urea and trichloroacetic acid [29, 32]. Other TH analogues may bind with proteins in a different manner. For instance, Lorenzini *et* al. recently observed that extraction with methyl tetra butyl ether (MTBE) was not able to improve the recovery of $3\text{-}T_1AM$ from serum, indicating $3\text{-}T_1AM$ binds with proteins in a covalent manner [40]. A standardized sample cleanup procedure is warranted. Additionally, a drawback of the HPLC-MS/MS based method is the necessity of relatively large tissue volumes, which makes it difficult to use it in small specimen such as experimental or clinical biopsies.

High resolution MS provides a full-scan spectrum of the THs in blood and tissue samples [36, 38]. The combination of HPLC with Q-TOF has received fast recognition since it is able to elucidate and screen target and non-target analytes from complex mixtures using extracted ion chromatograms (EIC) [36, 41]. This can be used to identify and characterize the co-eluents that induce matrix effects, which is a valuable information for improving sample clean-up procedures and chromatographic optimization.

Although it is of significant importance, only two methods were reported for TH analysis in human placenta using IA method or LC-MS/MS technique. The levels of T_4, T_3, rT_3 quantified by IA approach were 18.8, 0.026, and 1.70 ng/g fresh weight (fw), respectively. Their levels measured with LC-MS/MS were 11.8–53.6, 0.10–0.84 and 0.73–7.59 ng/g fw, respectively [42, 43]. LC-Q-TOF-MS methods are warranted. TH analysis in human breast milk was developed by IA approach [44]. LC-MS and LC-MS/MS methods are warranted.

The quantification methods for $3\text{-}T_1AM$ have been developed primarily using LC-MS/MS and IA technologies. Scanlan *et* al. firstly developed a semi-quantitative method to identify endogenous $3\text{-}T_1AM$ in rat brain. After extraction with 0.1 M perchloric acid and ethyl acetate, the fragments of $3\text{-}T_1AM$ were observed with LC-MS/MS [18]. In 2010, Saba *et* al. optimized the preanalytical procedures for $3\text{-}T_1AM$ in mouse blood and tissues using a weak cation

exchanger cartridge. In combination with LC-MS/MS, endogenous 3-T_1AM was quantified in serum and tissues such as heart, liver, kidney, skeletal muscle, stomach, lung, and brain [45]. The sample cleanup procedures are shown in Fig. 1.2. These two methods with minor modifications were applied for 3-T_1AM quantification.
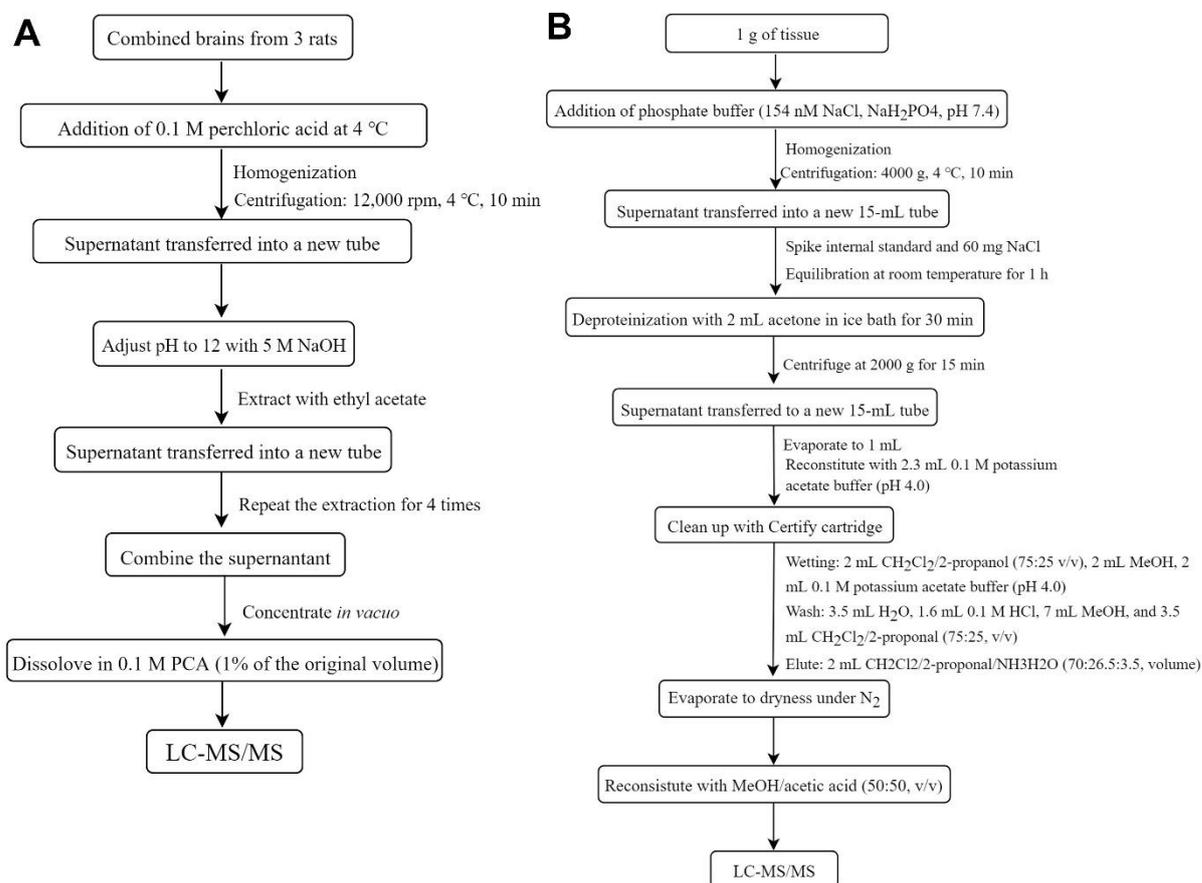
Fig. 1.2 Reported sample cleanup procedures for 3-T_1AM. (A) without cartridge [18] and (B) with Certify cartridge cleanup [45].

Ackermans *et al.* optimized a LC-MS/MS method using OASIS WCX cartridge cleanup as shown in Fig. 1.3 [46]. This method showed sufficient sensitivity with LODs of 0.25 nM in rat plasma and 0.30 pmol/g in rat tissues, respectively. Besides, Li *et al.* optimized sample cleanup with a weak cation exchanger cartridge (Bond Elute PCX) and determined with LC-Q-TOF-MS [37]. Hansen *et al.* employed a polymeric SPE cartridge for sample cleanup and determined with LC-MS/MS approach [32]. The sample type, extraction, equipment, analytes, and quantified concentration of these methods are summarized in Table 1.2.
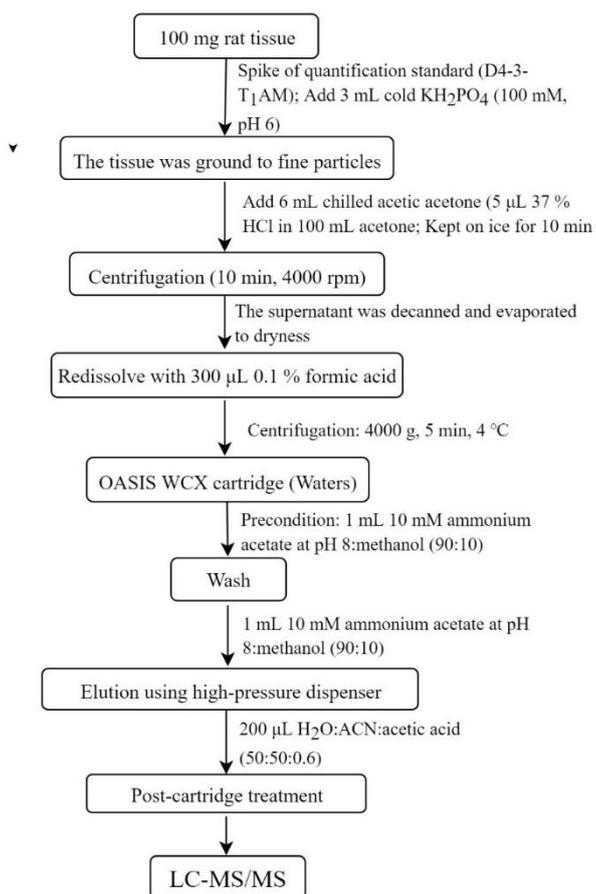
Fig. 1.3 Reported sample cleanup procedure for 3-T$_1$AM by Ackermans *et* al. [46]

As shown in Table 1.2, inconsistencies exist regarding the endogenous 3-T$_1$AM. Ackermans *et* al. and Li *et* al. did not find the existence of endogenous 3-T$_1$AM in rat and human tissues [37, 46]. Lerenzini *et* al. observed pitfalls in the quantification of 3-T$_1$AM in serum. For example, 3-T$_1$AM decrease exponentially in buffer containing fetal bovine serum (FBS), with a half-life time of 6–17 min, depending on FBS concentration [40]. Extraction with organic solvent (i.e., MTBE) was not able to improve 3-T$_1$AM recoveries.

Notably, deuterated 3-T$_1$AM (D4-T$_1$AM), which has been used as the quantification standard for 3-T$_1$AM, showed higher recovery than 3-T$_1$AM [40]. This indicates the 3-T$_1$AM level might be underestimated when employing D4-T$_1$AM as internal standard. Further studies are warranted to evaluate the recovery of ^{13}C-labeled 3-T$_1$AM and as internal standard.

Table 1.2 LC-MS/MS technologies for 3-T_1AM and T_0AM analysis reported up to now [19].

Sample	Extraction	Equipment	Analytes	Concentration	IS	Comments	Ref.
Rat brain, guinea-pig brain, heart, liver, and blood	Method A	LC-MS/MS	3-T_1AM, T_0AM	—	—	Qualitative analysis based on single transition	[18]
Rat heart	Method A	LC-MS/MS	3-T_1AM	68 pmol/g (1–210 pmol/g)	D4-3-T_1AM		[47]
Rat heart, liver, kidney, muscle, stomach, lung, and serum	Method B	LC-MS/MS	3-T_1AM, T_0AM	3-T_1AM: 5.61–92.9 pmol/g in tissue and 0.30 pmol/L in serum; T_0AM: 0.27, 6.01, and 20.7 pmol/g in muscle, kidney, and liver, 0.04 pmol/L in serum.	D4-3-T_1AM, D4-T_0AM		[45]
Male CD-1 mice serum	Method B	LC-MS/MS	3-T_1AM	0.05 nM	D4-3-T_1AM	Periphery injection of MAO inhibitor clorgyline.	[48]
Outbred female CD-1 mice: liver, white adipose tissue,	Method B	LC-MS/MS	3-T_1AM	0.49–19.8 pmol/g	D4-3-T_1AM		[49]

muscle, heart							
Male C57BL/6J mice	Method B	LC-MS/MS	3-T_1AM	2.4 pmol/g	D4-3-T_1AM		[50]
Djungarian hamster serum	Method A	LC-MS/MS	3-T_1AM	5.86–5.95 nM	D4-3-T_1AM	3 hours following carrier injection	[51]
Human plasma, human thyroid tissue, rat plasma, rat liver	Ackermans *et al.* [46]	LC-MS/MS	3-T_1AM	< LOD	D4-3-T_1AM	No endogenous 3-T_1AM was found	[46]
Human blood	Method B	LC-MS/MS	3-T_1AM	0.15–0.20 nM	D4-3-T_1AM		[45]
Human blood	Method B	LC-MS/MS	3-T_1AM	0.22 ± 0.01 nM	D4-3-T_1AM	Diabetic patients: 0.232 ± 0.014 nM; Nondiabetic patients: 0.203 ± 0.006 nM	[31]
Human placenta	Li *et al.* [37]	LC-Q-TOF-MS	3-T_1AM	< LOD	$^{13}C_6$-3,3'-T_2	No endogenous 3-T_1AM was found	[37]
Tadpole serum	Hansen *et al.* [32]	LC-MS/MS	3-T_1AM	3.11 ± 1.18 nM	$^{13}C_6$-3,3'-T_2	Quantification frequency of 3-T_1AM was 30%	[32]

Hoefig *et* al. generated mouse monoclonal 3-T_1AM antibodies and established a monoclonal antibody-based chemiluminescence immunoassay for 3-T_1AM analysis in serum [52]. Cross-reactivities and quality assessment parameters proved the suitability of this approach. With this method, the median serum concentration of 3-T_1AM was quantified to be 66 ± 26 nM in healthy adult humans [52], and 15 nM in patients after cardiac surgery [53]. Langouche *et* al. adapted and optimized this method and quantified that the serum 3-T_1AM of stationary and ambulatory patients was 5 nM [54]. These values were much higher than those measured with LC-MS/MS approach, which were 0.15–0.20 nM in human serum [45], and 0.22 nM in serum of stationary and ambulatory patients [31]. One plausible reason is the binding of 3-T_1AM to serum proteins which prevent rapid and complete release during sample cleanup [40]. Using 3-T_1AM-affinity chromatography, Roy *et* al. demonstrated that 3-T_1AM is specifically binding with apoB-100 containing low-density lipoproteins with an equilibrium dissociation constant of 17 nM and a 3-T_1AM/apoB-100 stoichiometry of 1:1 [55]. This partially explains the decrease of 3-T_1AM in buffer containing FBS as described above. Another reason might be the limited selectivity of IA approach.

Additional attempts in TAM analysis includes an electrochemical sensor for T_0AM via molecular imprinted polymers. The LOD and LOQ of this method were 0.081 and 0.27 μM, respectively [56].

1.3 Persistent organic pollutants

Persistent organic pollutants (POPs) are a wide variety of chemicals that have raised global concern because of their potential for long-range transport, resistance to degradation, bioaccumulation and biomagnification in the ecosystems, as well as their significant negative effects on human health and environment. Many products used in our daily lives may contain POPs to improve product characteristics. POPs are emitted to the environment from these products and unintentional by-products of many processes such as incomplete combustion and traffic [57]. Humans are exposed to POPs through diet, air, house dust, and drinking water [58]. POPs are lipophilic, bioaccumulate in wildlife and humans, and could biomagnify up the food chain.

In 2004, the Stockholm Convention on Persistent Organic Pollutants was ratified by

governments to decrease environmental and human exposure to 12 POP substances including aldrin and dieldrin, chlordane, dichlorodiphenyltrichloroethane (DDT), endrin, mirex, heptachlor, hexachlorobenzene, PCBs, toxaphene, dioxins and furans, which were called the "Dirty Dozen". Since then, more compounds have been included into the list to reduce or cease their production. As shown in Table 1.3, Annex A includes the POPs whose production must be eliminated. Annex B include the POPs whose production must be restricted. Annex C include those that the unintentional releases should be reduced.

Table 1.3 All POPs listed in the Stockholm Convention.

Category	POPs
Annex A (Elimination)	Aldrin, chlordane, chlordecone, decabromodiphenyl ether (commercial mixture, c-decaBDE, dieldrin, endrin, heptachlor, hexabromobiphenyl, hexabromocyclododecane (HBCDD), hexabromodiphenyl ether and heptabromodiphenyl ether, hexachlorobenzene (HCB), hexachlorobutadiene, α hexachlorocyclohexane, β hexachlorocyclohexane, lindane, mirex, pentachlorobenzene, pentachlorophenol and its salts and esters, PCB, polychlorinated naphthalenes, short-chain chlorinated paraffins (SCCPs), technical endosulfan and its related isomers, tetrabromodiphenyl ether and pentabromodiphenyl ether, toxaphene
Annex B (Restriction)	DDT, perfluorooctane sulfonic acid, its salts and perfluorooctane sulfonyl fluoride
Annex C (Unintentional production)	HCB, hexachlorobutadiene (HCBD), pentachlorobenzene, PCB, PCDD, PCDF, polychlorinated naphthalenes

Despite their decreased release, exposure to these compounds continues due to their resistance to environmental degradation [59]. For example, PBDEs have half-lives up to 12 years in human [60]. POPs are global contaminants that have been detected in the environment and human adipose tissue, serum and breast milk samples all over the world [61-64].

The most commonly encountered POPs include polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs),

polybrominated dibenzo-*p*-dioxins and furans (PBDD/Fs), organochlorinated pesticides (OCPs), organotin compounds (OTCs), and polyfluorinated alkyl substances (PFASs). The chemical structures of some of the POPs are shown in Fig. 1.4. PBDEs are mainly used as flame retardants in plastics, foam, and textiles in clothing, electronic components, furniture and cars. PCBs are used as coolants/insulating fluids, electrical wiring and electronic components, pesticides, flame retardants, hydraulic fluids, adhesives, cutting oil, sealants, wood floor finishes, paints, and in carbonless copy paper. Dioxins are mainly released by municipal waste incineration, metal smelting, coal burning plants, diesel trucks, burning treated wood and trash burn barrels, and land application of sewage sludge. Dioxins are also generated in bleaching fibers for papers and textiles. OCPs were used to control insects and termites, fungi, mosquito, rodent, and used as flame retardant. OTCs are widely used as polyvinyl chloride (PVC) stabilizers, biocides, or antifouling paints.
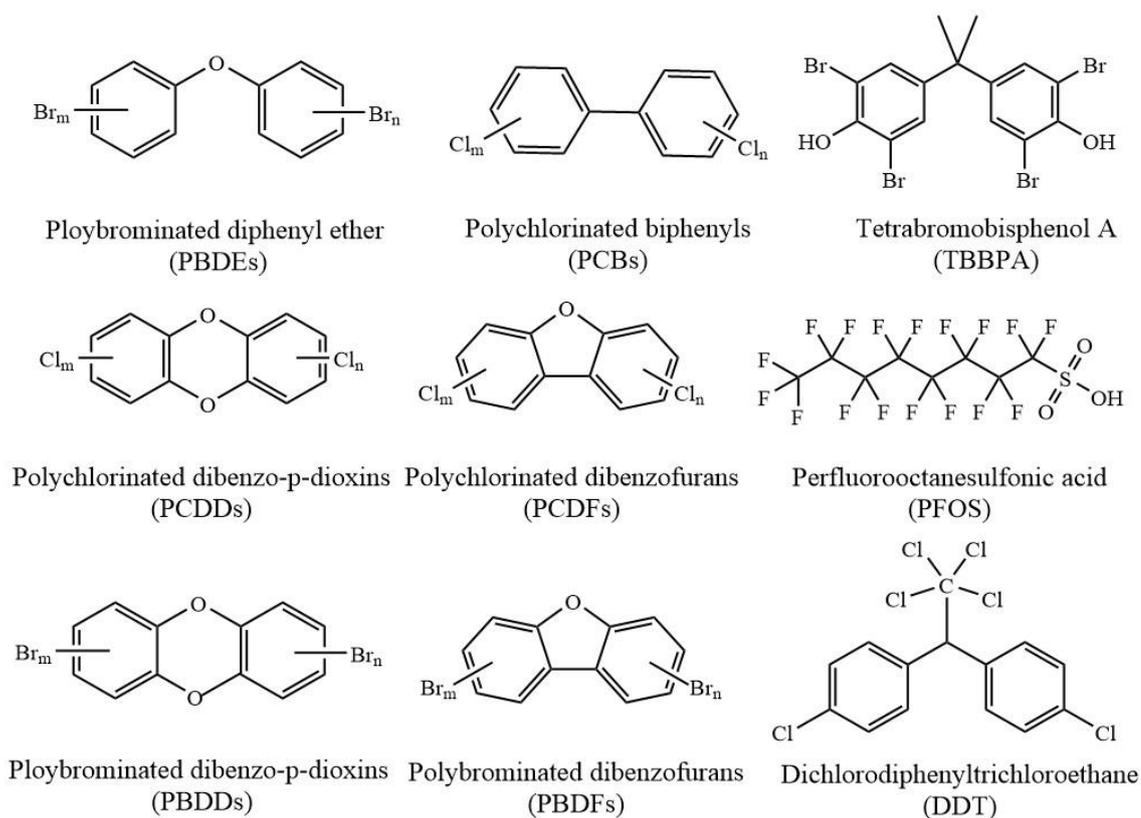
Fig. 1.4 Molecular structures of some of the persistent organic pollutants (POPs)

Some POPs can be metabolized in the environment or *in vivo*, including hydroxylation, dehalogenation, etc. For example, PCBs and PBDEs can be oxidized by cytochrome P450

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enzymes, resulting in OH-PCBs and OH-PBDEs, respectively [65, 66]. These compounds are of concern because some of them may accumulate in human serum at levels similar to or even higher than the parent compounds [67]. Other studies revealed that OH-PBDEs showed higher potencies of thyroid disruption than PBDEs [68].

1.4 Thyroid-disrupting properties of environmental chemicals

Certain POP congeners have similar chemical structures to THs leading to concerns about their potential thyroid-disrupting properties. The mechanisms involved are complex and diverse:(1) POPs may disrupt the activity of the thyroid gland by interfering with THS-receptor, sodium iodide symporter, thyroid peroxidase, and other proteins on the thyrocyte; (2) POPs may competitively bind to TH binding proteins, i.e., thyroid-binding globulin, TTR and TR. For example, *in vitro* studies demonstrated that low-brominated OH-PBDEs are structurally similar to THs and can competitively bind with TR [68, 69]. Competitively binding of POPs with TH binding proteins may result in increased metabolism of THs; (3) POPs may affect the peripheral TH metabolism and clearance by interference with the enzymes such as CYPs, UGTs, SULTs, etc. [70].

Reduced T_4 was observed in dams and offspring of Wistar rats with a single low dose of BDE-99 [71]. A substantial body of epidemiologic studies also reported decreased THs with increasing exposure of POPs [72-78]. Besides, Hoffmann *et* al. observed that higher levels of BDE-209 and tris(2-chloroethyl) phosphate in dust were positively associated with the odds of papillary thyroid cancer in an American cohort [79]. Han *et* al. also reported positive associations between the odds of thyroid disease and the sum of 17 POPs in a population in East China [80]. In contrast, others observed positive or nonsignificant associations [81, 82]. The inconsistence is probably the results of random error given the intraindividual variability in THs specimen type. For example, Stapleton *et* al. [81] and Zota *et* al. [82] employed serum samples from women during pregnancy when marked fluctuations in HPT axis homeostasis occur [83], while some other studies were collected from males or non-pregnant females. Another possibility is that the relationship between POPs and THs may vary by exposure level. THs act at quite low concentrations (free serum T_4 level: 8–20 ng/L [84]) while low-dose

effects and non-monotonic responses are remarkably common in studies of endocrine-disrupting compounds (EDCs) [85]. Abdelouahab *et* al. observed significant decrease in total T_4 (TT_4) and total T_3 (TT_3) in lambs following low-dose exposure of BDE-47 [86]. A meta-analysis suggested that the relationship between TT_4 and PBDEs might be negative when serum PBDEs < 35 ng/g, while positive at higher exposure levels [87]. Other probable reasons include the presence of different pollutant mixtures, different exposure levels, varying timing of sample collection, and the differences in living environment and life styles of populations.

TT_4 in placenta mainly originates from maternal free T_4, while TT_3 and total rT_3 (TrT_3) are derived entirely from placental and foetal metabolism of T_4 [88]. Disrupting the TH-protein binding and metabolism in placenta may be of significance. TTR plays a crucial role in transferring free T_4 across the placenta [70]. Binding of certain POPs to TTR may facilitate the transport of these compounds, while reducing T_4 delivery to the fetus. Only one study was performed in placenta on the associations of THs with PBDEs [42], while no research is available for PCBs, PCDD/Fs, OTCs, and OCPs. It is therefore worthwhile to conduct a comprehensive analysis by including as many POPs as possible to have a complete overview of the influence of *in utero* POP exposures on THs. POPs are transferred to the fetus across the placenta [89, 90]. Passive diffusion and/or active uptake are involved in this process. The placenta can also act as a repository for these lipophilic chemicals. Thus, there is a risk for disruption of the fetal development because POPs in placenta may affect the amount of THs delivered to the fetus. This is particularly important during early pregnancy when the fetus depends solely on maternally-derived THs [91].

Besides, the low detection frequencies of POPs in serum of background exposed populations may also play a role for the inconsistences [92]. Breast milk is a complex and constantly changing mixture of endogenous and exogenous substances including THs and POPs [44, 63]. Due to its high lipid content, breast milk has been increasingly employed as a suitable specimen to monitor POP exposures and associated outcomes [63, 93]. Several studies assessed the associations of POPs in milk and serum TH parameters [94, 95]. However, to our knowledge, no study has been conducted to evaluate the associations of THs and POPs both measured in human breast milk.

1.5 Association of POP exposures with type 2 diabetes

Traditional lifestyle risk factors such as physical inactivity, smoking and obesity are not sufficient to explain the increasing trend of current epidemic of type 2 diabetes (T2D). In 2006, Lee *et* al. observed associations between POPs and diabetes for the first time in U.S. adults. In this study, POPs including HpCDD, OCDD, DDE, PCB-153, oxychlordane, trans-nonachlor showed significant positive associations with diabetes [96].

Since then, a flurry of studies have been conducted to assess the relationship between POPs and T2D and related conditions such as type 1 diabetes (T1D), gestational diabetes mellitus (GDM), insulin resistance, obesity, metabolic syndrome, and prediabetes. A summary is given in Table 1.4.

The mechanisms involved in the association between T2D and POPs remain unclear. Endocrine-disruption and mechanisms related to mitochondrial dysfunction are proposed to play a role [97-99]. Besides, the evidence of numerous epidemiologic studies was limited, probably because of selection bias, low number of study participants, and cross-sectional study design.

1.6 Association of THs with GDM

GDM is defined as any degree of glucose intolerance that is first recognized during pregnancy. The prevalence of GDM has been steadily increasing in many countries, including China. GDM may lead to serious adverse maternal outcomes such as high cesarean section rate and preeclampsia and detrimental infant outcomes such as macrosomia, infant respiratory distress syndrome, and neonatal hypoglycemia. GDM also increases the long-term risk of T2D, obesity, and metabolic syndrome for both mothers and infants [100]. The metabolic changes and increased cardiovascular risk described in the metabolic syndrome are very similar to the changes seen in hypothyroidism. Epidemiologic studies have also demonstrated that increased cardiovascular disease (CVD) events, blood pressure, dyslipidemia and mortality in subjects with subclinical as well as overt hypothyroidism, proposing substantial impact of thyroid insufficiency on atherosclerotic vascular changes in a graded manner [101].

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Table 1.4 Summary of previous reports of the associations between POP exposures and T1D, autoimmunity, T2D, GDM, and childhood obesity.

Metabolic Syndrome	POPs			
	PCB	Dioxins	OCP	Others
T1D			Malathion [102], o,p'-DDE [102], o,p'-DDD [102], endrin [102], o,p'-DDT(-) [102], profenofos(-) [102], chlorpyrifosmethyl(-) [102]	PFOS [103], PFOA(-) [104], PFOS(-) [104], PFNA(-) [104], PFHxS (-) [104],
Autoimmunity		TCDD [105]		Phthylates [106], trichloroethylene [107]
T2D	Total PCB [108], PCB-74 [108], PCB-99 [109], PCB-105 [110], PCB-118 [109, 110], PCB-138 [109], PCB-146 [111], PCB-153 [109, 112], PCB-156 [109], PCB-157 [113], PCB-163 [109, 113], PCB-170 [109], PCB-180 [109, 111], PCB-183 [109], PCB-187 [109], nondioxin-like PCBs (PCB-28, -52, -101, -118,	HpCDD [96], dioxin-like PCBs [115], TCDD [116]	p,p'-DDT[117], o,p'-DDT [114], p,p'-DDE [112],o,p'-DDE [114], o,p'-DDD [114], p,p'-DDD [114], oxychlordane [96], α-HCH [114], β-HCH [117], γ-HCH [114], δ-HCH [114], transnonachlor [96], HCB [108], CB-153 [118], heptachlor epoxide, hexachlorobenzene, Mirex [109]	PBB-153 [119]

	-138, -153, and -180) [114]		
GDM	PCBs (≥6Cl) [120], total PCBs [120], dioxin-llike PCBs (PCB-118, -156), non-dioxin like PCBs (-138, -153, -170, -180) [120],	OC [121]	PFNA [120], PFOA [120, 122], PFHpA [120], PFDoDA [120], BDE-47 [120], BDE-153 [120, 123], BDE-154, BDE-183, DMP(-) [124], DMTP(-) [124], PFHxS [124],
Childhood Obesity	PCBs (-138, -153, and -180) [125], PCB-153 [126]	o,p'-DDT [127], p,p'-DDT [127], p,p'-DDE [125]	MnBP, MEP [128], MiBP, MEHHP [129], MEOHP [129], DEHP [129], MEHP(-) [130], BPA [131], triclosan [132], pentaBDEs (-47, -99, -100, -153) [126]

Abbreviations: PBB, polybrominated byphenyl; OC, organochlorine compounds; DMP, dimethylphosphate; DMTP, di- methylthiophosphate; MnBP, mono-n-butyl phthalate; MEP, monoethyl phthalate; MiBP, mono-isobutyl phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MEOHP, mono(2- ethyl-5-oxohexyl) phthalate; DEHP, di-2-ethylhexyl phthalate; MEHP, mono-(2-ethylhexyl) phthalate

Other studies have demonstrated close associations between thyroid insufficiency and clusters of metabolic abnormalities such as obesity and lipid profiles, observed even within physiological ranges [101]. Shin *et* al. found that free thyroxine (FT$_4$) levels were negatively associated with body mass index, waist circumference, triglyceride, c-reactive protein, and HOMA-IR and positively with high-density lipoprotein cholesterol in both genders [133]. THs might be associated with metabolic syndromes such as obesity and T2D [1]. THs can increase glucose uptake through enhanced expression of glucose transporter type 4 (GLUT4). The interaction of TRβ and PI3 kinase mediates the glucose uptake. It is noteworthy that maternal thyroid dysfunction during pregnancy may be related with GDM. For example, Karakosta *et* al. observed a 4-fold increase of risk for GDM in mothers with high TSH and thyroid autoimmunity in early pregnancy [134]. Therefore, it is of importance to analyze the association between THs and the odds of GDM.

1.7 Placental transfer and distribution of nutrients and toxicants

Placenta and umbilical cord are highly specialized organs that are fundamental for the development and maintenance of pregnancy. They work as a regulator and transport system for nutrients and essential hormones [91, 135]. Passive diffusion, active transport, facilitated diffusion, filtration and pinocytosis are involved in this process [136]. Environmental contaminants are also transferred across the placenta due to chemical similarities with nutrients, or simply as a result of passive diffusion. The developing fetus is particularly susceptible to these abnormal stimuli because the organs, excretory system and blood-brain barrier are not fully developed. Recent studies indicate that *in-utero* exposure may disrupt the homeostasis of hormones and elements in the fetus [42, 73, 137], resulting in severe repercussions for newborns and late adult deleterious effects [138, 139].

During pregnancy, maternal THs can be transferred to the fetus through placenta to support normal fetal brain development [140]. This is particularly important during early pregnancy, when the fetus relies solely on maternally-derived THs [91]. The human thyroid system is susceptible to disruption by endogenous (e.g., autoantibodies) or exogenous (e.g., iodine) factors, by interfering with the sodium-iodide symporter, TH metabolism, receptors, and TH transport [141]. Maternal thyroid hormones could directly affect the development of the

offspring's central nervous system (CNS) [142] and the development of the placenta [140] from the first trimester of pregnancy. Even minor changes in maternal TH circulation can lead to various adverse outcomes, including miscarriage, intrauterine growth retardation, hypertensive disorders, preterm delivery, and a decreased child IQ [143].

Placenta and umbilical cord tissues have been increasingly used as biomarkers to assess the fetal nutrients and environmental exposure [144, 145]. These samples are easily accessible, require no invasive procedure, and offer possibilities for monitoring the pollutant burden exerted on both the mother and the fetus [93]. The term human placenta, composed of fetal and maternal portions, is discoid in shape with a diameter of 15–20 cm and a thickness of 2–3 cm [146]. The umbilical cord in a full-term neonate is about 55 cm long and about 2 cm in diameter [147]. In 1987, Manci *et* al. observed correlations between the locations of the sampling sites and the levels of Fe, Zn, Cu, and Ca [148]. Later studies also reported heterogeneity in the distribution of Fe, Hg, Cu, Ca, Cd, and Pb in placenta [146, 149, 150]. However, little is known about the distribution of other metals (e.g., As, Cr, Hg, Se, Mn, K, Mg, Na, Al, etc.), hormones, and POPs, which have been proved to interfere with pregnancy. Besides, the regional variation of these constituents in umbilical cord remains unexplored.

Due to the potential heterogeneity of various chemicals in placenta and umbilical cord, obtaining a representative placenta sample for both essential and toxic chemicals is of difficulty [93]. To avoid potential bias, some studies homogenized the entire placenta [151, 152], which is cumbersome and increases the risk of sample contamination. Other studies collected the same part of placenta and umbilical cord [153, 154], or collected random samples from unspecified sites within the placenta and umbilical cord [155]. However, the validity of this procedure needs to be tested.

1.8 Exposome Wide Association Study (EWAS) and sample pooling approach

It has been indicated that 70–90% of the diseases are probably due to environmental reasons while genetics account for only about 10% of diseases [156]. In 2005, shortly after the

sequencing and mapping of the human genome, Christopher P. Wild proposed a cutting-edge concept of "exposome", which refers to the totality of endogenous and exogenous exposures from the conception and throughout the lifespan [157]. Later Stephen M. Rappaport further advocated this concept [158-160].

We analyzed the targeted exposomics of human placenta, umbilical cord, cord blood, and breast milk based on targeted biomonitoring. As shown in Fig. 1.5 &1.6.
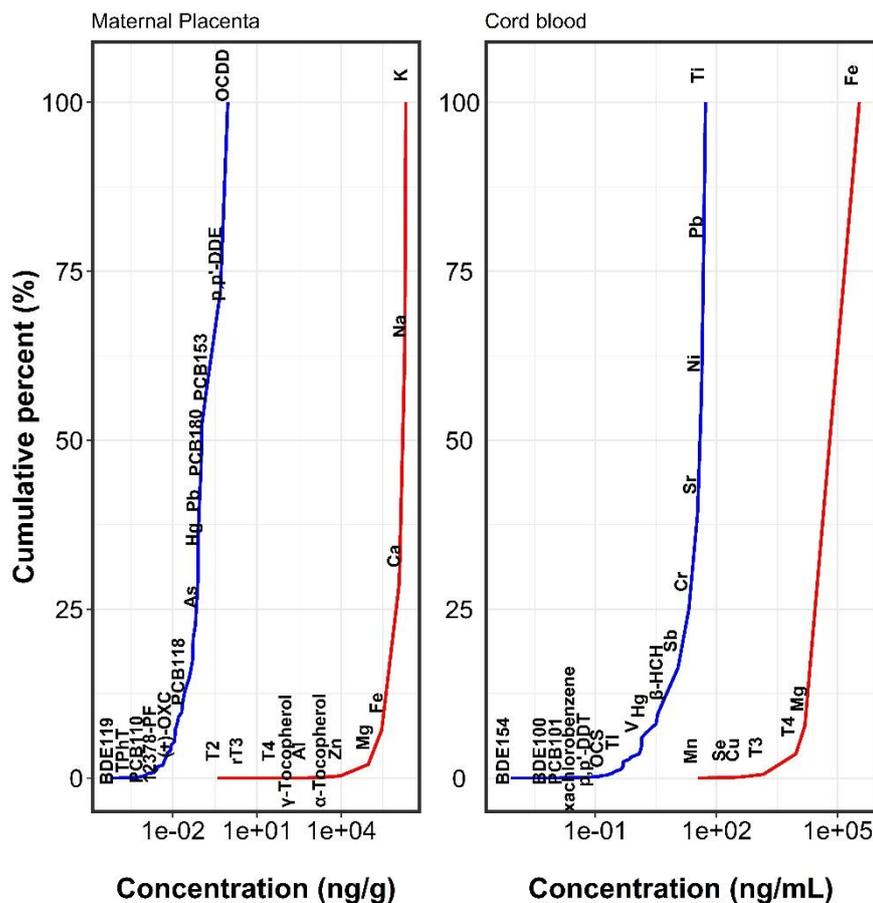
Fig. 1.5 Human placenta and cord blood exposomics.

Exposomics is the study of the exposome and relies on the assessment of internal and external exposures. Traditional targeted biomonitoring based on isotope-dilution and LC-MS/MS provides high sensitivity and selectivity, which, however, is laborious, expensive, and requires relatively large sample size. For example, > 20 mL serum is essential to quantify the 250–300 currently biomonitored chemicals, which can be limiting for certain age groups under study [161]. Untargeted exposomic approach provides a means to improved exposure assessment compared with traditional biomonitoring. This methodology is able to reveal the

exposures and the metabolic consequences of exposures including nonchemical stressors such as psychosocial stress. Untargeted biomonitoring method using high-resolution mass spectrometry is a promising approach in exposomics, which is able to measure more than 1500 metabolites with a relatively small amount of specimen (≤ 100 μL serum) [162].
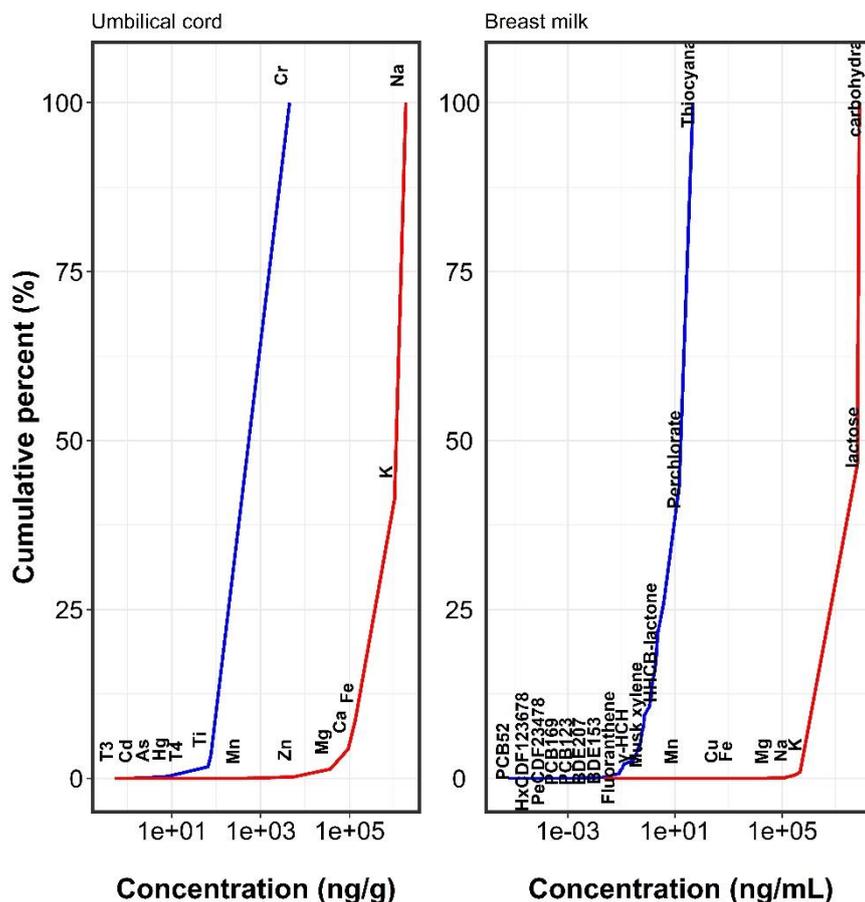
Fig. 1.6 Human umbilical cord and breast milk exposomics.

To reduce complexity in explorative assessment, Shen *et al.* proposed a sample pooling approach [163]. This is based on the hypothesis that if certain chemicals show significant differences in case and control populations, then they should be significantly different in pooled cases and controls. Individual samples in case and control populations were randomly grouped, then the analytes were quantified in the pooled cases and pooled controls. In this way, the sample number in each group reduced adequately while the sufficient sample size was obtained. Besides, according to the general central limit theorem, the distribution of the chemical concentrations in each subpopulation would tend to be normal [164].

1.9 Objectives addressed in this thesis

This dissertation focuses on the human exposomics of POPs in relation to endogenous biomarkers, namely THs and monoamines. Ultra-trace analytical methods were developed and optimized employing state of art UPLC-Q-TOF-MS and HPLC-QqQ-MS in combination of isotope dilution technology. Further, the associations of THs with environmental exposures and GDM were evaluated. The framework of this thesis is outlined as following (see also Fig. 1.7):

(1) Development of TH determination methods with UPLC-Q-TOF-MS and HPLC-MS/MS in various matrices;

(2) Method development for the quantification of endogenous 3-T$_1$AM in mouse blood and tissues;

(3) Assessment of the thyroid-disrupting properties of POPs using human placenta samples from the Danish EXPORED cohort, and human breast milk samples from the German LUPE cohort;

(4) Evaluation of the placental distribution of various nutrients and toxicants;

(5) Evaluation of the potential association between GDM and placental THs;

(6) Application of the sample pooling strategy in EWAS.
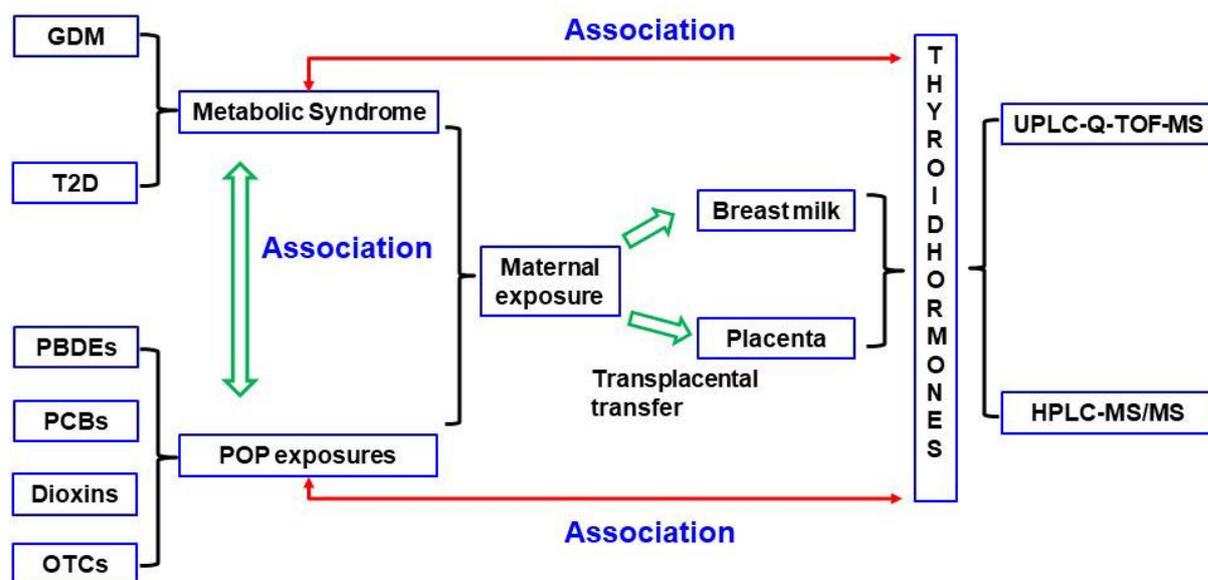
Fig. 1.7 Framework shows the research topics and contents in this thesis.

Chapter 2 Determination of thyroid hormones in placenta using isotope-dilution liquid chromatography quadrupole time-of-flight mass spectrometry

Abstract

THs of maternal origin are of great significance for ensuring the normal fetal development. In this chapter, we analyzed the concentrations of T_4, T_3, rT_3, $3,3'-T_2$, $3,5-T_2$, T_1 and $3-T_1AM$, in placenta using isotope dilution LC-Q-TOF-MS. We optimized the method using isotopically labeled quantification standards ($^{13}C_6-T_4$, $^{13}C_6-T_3$, $^{13}C_6-rT_3$ and $^{13}C_6-T_2$) and recovery standard ($^{13}C_{12}-T_4$) in combination with solid-liquid extraction, liquid-liquid extraction and solid phase extraction. The linearity ranged from of 0.5 to 150 pg/uL with R^2 values > 0.99. The MLODs were 0.01–0.2 ng/g, while the MLOQs were 0.04–0.7 ng/g. The spike-recoveries for THs (except for T_1 and T_1AM) were from 81.0% to 112%, with a coefficient of variation (CV) of 0.5–6.2%. The intra-day CVs and inter-day CVs were 0.5%–10.3% and 1.19%–8.88%, respectively. Concentrations of the THs were 22.9–35.0 ng/g T_4, 0.32–0.46 ng/g T_3, 2.86–3.69 ng/g rT_3, 0.16–0.26 ng/g T_2, and < MDL for other THs in five human placentas, and 2.05–3.51 ng/g T_4, 0.37–0.62 ng/g T_3, 0.96–1.3 ng/g rT_3, 0.07–0.13 ng/g T_2 and < MDL for other THs in five mouse placentas. The presence of T_2 was tracked in placenta for the first time. This method with improved selectivity and sensitivity allows comprehensive evaluation of TH homeostasis.

Author contribution:

Zhong-Min Li was involved in the study design, measurement performance, data

acquisition and interpretation, and manuscript preparation. Florian Giesert and Daniela Vogt-Weisenhorn collected mouse placenta samples. Katharina Maria Main, Niels Erik Skakkebæk, Hannu Kiviranta, Jorma Toppari, Ulla Feldt-Rasmussen, and Heqing Shen were involved in study design and manuscript review. Karl-Werner Schramm and Meri De Angelis were involved in the study design, sample collection, data interpretation, and manuscript review.

2.1 Introduction

This chapter introduces a method for the determination of THs in placenta. We used isotope dilution liquid chromatography quadrupole time-of-flight mass spectrometry. We optimized the HPLC and MS conditions, and sample cleanup procedures. The methods were evaluated with linearity range, instrument limits of detection (ILOD) and quantification (ILOQ), method limits of detection (MLODs) and quantification (MLOQs), spike-recoveries, matrix effects, intra-day and inter-day variations. Finally, this method was applied for TH quantification in human and mouse placenta, and other matrices such as mouse serum/plasma, kidney, and liver.

2.2 Experimental

2.2.1 Materials and chemicals

Fig. 2.1 shows the molecular structures of the investigated THs, quantification and recovery standards. Individual certified stock solutions of T_4, T_3, rT_3, $^{13}C_6$-T_3, and $^{13}C_6$-rT_3 at 100 µg/mL (dissolved in MeOH containing 0.1 M NH_4OH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 3,3'-T_2, T_1 and 3-T_1AM were from Sigma-Aldrich (St. Louis, MO, USA). 3,5-T_2 and $^{13}C_6$-3,5-T_2 were from Santa Cruz Biotechnology (Dallas, Texas, USA). $^{13}C_6$-T_4 and $^{13}C_{12}$-T_4 were purchased from Cambridge Isotope Laboratories (Andover, MA). Primary individual stock solutions of these compounds (50 µg/mL for 3,3'-T_2, T_1, $^{13}C_6$-T_4 and $^{13}C_{12}$-T_4, 100 µg/mL for 3,5-T_2, 3-T_1AM and $^{13}C_6$-3,5-T_2) were prepared in MeOH containing 0.1 M NH_4OH.

6-Propyl-2-thiouracil (PTU) was from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and solvents were of ACS grade or LC-MS grade. Distilled water was obtained using

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a water distillation purification system and was used for the preparation of all aqueous solutions. Finally, an antioxidant solution consisting of 10 mg/mL citric acid monohydrate, L-(+)-ascorbic acid and R, R-dithiothreitol was prepared daily in water.

Target compounds
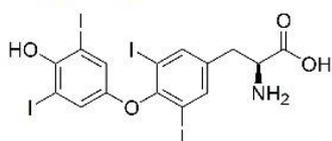
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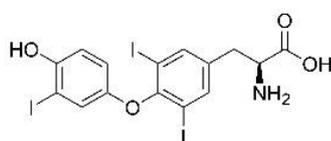
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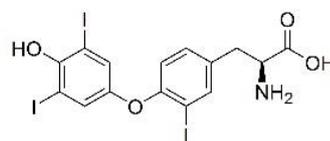
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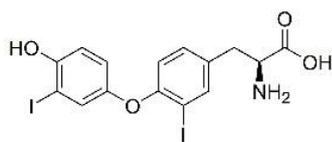
3,3'-Dioiodothyronine (3,3'-T₂)
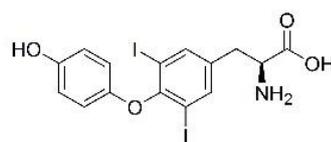
3,5-Dioiodothyronine (3,5-T₂)
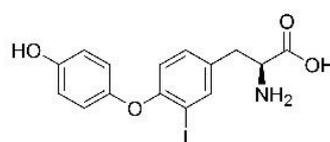
3-Iodothyronine (T₁)
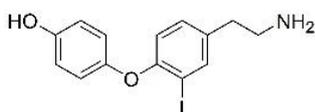
3-Iodothyronamine (T₁AM)

Internal standards
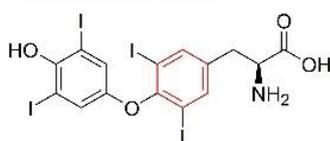
¹³C₆-Thyroxine (¹³C₆-T₄)
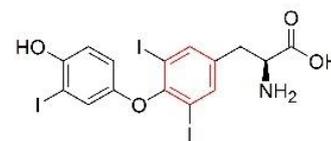
¹³C₆-3,3',5-Triiodothyronine (¹³C₆-T₃)
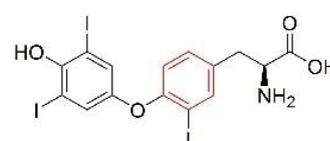
¹³C₆-3,3',5'-Triiodothyronine (¹³C₆-rT₃)
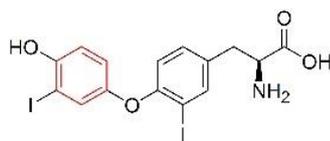
¹³C₆-3,3'-Diiodothyronne (¹³C₆-T₂)

Recovery standards
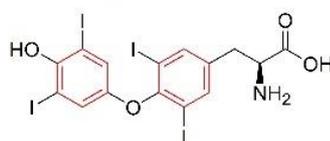
¹³C₁₂-Thyroxine (¹³C₁₂-T₄)

Fig. 2.1 Molecular structures of the THs, internal standards, and recovery standard

HybridSPE®-Phospholipid cartridges (30 mg/1 mL) were from Sigma-Aldrich (St. Louis, MO, USA), SampliQ OPT cartridges (60 mg/3 mL), Bond Elut Plexa cartridges (60 mg/3 mL), Bond Elut Plexa PAX cartridges (60 mg/3 mL) and Bond Elut Plexa PCX cartridges (60 mg/3

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mL) were all obtained from Agilent Technologies (Santa Clara, CA, USA).

2.2.2 Tissue collection

We obtained the human placenta samples from a birth cohort study conducted in Finland and Denmark as described earlier [165, 166]. The study was handled under the guidelines of the Finnish ethics committee (7/1996) and Danish ethics committee (kF01-030/97).

C57BL/6J mice were obtained from Charles River Laboratories Inc. (Kisslegg, Germany). The animals were housed in a room at 22 °C under a normal 12-h light: 12-h dark cycle, with regular food and water available *ad libitum*. The mice were killed via cervical dislocation. Placentas were dissected, weighted, snap-frozen in liquid nitrogen and kept at −80 °C. The animal studies were handled under the guidelines of the District of Upper Bavaria, Germany. In order to reduce the mice sacrificed, we used human placenta for method development.

2.2.3 Sample preparation

(1) Placenta sample preparation

As shown in Fig. 2.2, a 500-mg portion of wet placenta sample was weighted and placed into a 15-mL polypropylene centrifuge tube. After adding 500 μL of MeOH, the sample was homogenized by ultrasonication (Bandelin Electronics, Berlin, Germany) on ice. Next, standards (target analytes and quantification standards) were spiked. Samples fortified with THs were used to optimize and validate the sample clean-up procedure.

For TH extraction and protein precipitation, 1 mL $CHCl_3$ was added, vortexed vigorously, and the sample was kept on ice for 60 min. Afterwards, the mixture was centrifugated at 7,000 rpm (5,478×g) for 10 min, and the liquid portion was decanted to another 15-mL tube. This process was repeated twice and the liquid was combined. Thereafter, 800 μL of 0.05% (w/w) $CaCl_2$ solution was added and vortexed. The homogenate was centrifuged (7,000 rpm, 10 min), and the supernatant was decanted into a new centrifuge tube containing 300 μL of antioxidant solution. The extraction process was repeated twice. H_3PO_4 was added into the combined extracts to reach a concentration of 2%. After vortex, the solution was further cleaned-up through Bond Elut Plexa PCX cartridges conducted on a vacuum manifold (Supelco, Bellefonte, PA, USA). The SPE cartridges were preconditioned with 1.5-mL MeOH and 1.5-mL H_2O. Thereafter, samples were extracted, and immediately washed with 2-mL 2% formic acid and

2-mL MeOH: ACN (1:1, v/v). A 1-min drying of the cartridges was performed using the vacuum pump. Analytes were then eluted into a vial with 1 mL of 5% NH_4OH (28–30%) in MeOH: ACN (1:1, v/v). The elute was evaporated to dryness under an atmosphere of N_2 at 40 °C. Afterwards, the residue was dissolved in 60 μL of 10 ng/mL $^{13}C_{12}$-T_4 in ACN: H_2O (2:8, v/v) containing 0.1% formic acid, vortexed vigorously, and transferred to an LC-vial. Finally, 5 μL aliquot was injected into the chromatographic system.
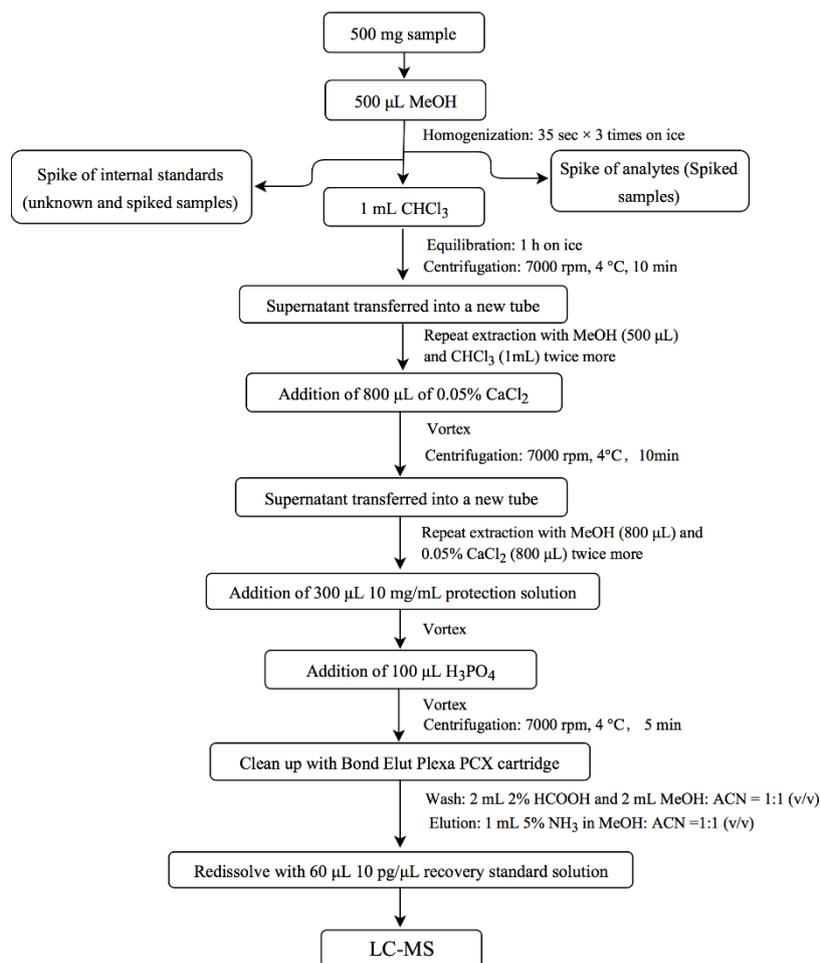
Fig. 2.2 Schematic diagram shows the optimized sample treatment procedure.

2.2.4 Instrumentation

The chromatographic optimization was based on a previous method [36] with some modifications. A nanoAcquity UPLC system (Waters, Milford, USA) was connected to a quadrupole time-of-flight (Q-TOF2) mass spectrometer (Waters Micromass, Manchester, UK). Samples were separated on an HSS-T3 micro-scale column (300 μm i.d.×150 mm length, 1.8 μm particle size) from Waters (Milford, MA, USA). The stationary phase of this column was

trifunctional C18 Alkyl phase bonded High Strength particle. The mobile phases of the gradient HPLC method were water (A) and ACN (B) each containing 0.1% formic acid (v/v). Formic acid was selected as the mobile phase additive over ammonium formate and acetic acid because it showed better peak shape, peak separation and signal intensity. For organic solvent, ACN was preferred over MeOH because of lower back-pressure. The following gradient program was adopted: 5% B kept for 3 min, ramped linearly to 30% B in 0.5 min, then gradually increased to 38% B in 2 min and kept for 1 min, followed by an increase to 40% B in 0.5 min and held for 2.5 min, then increased to 100% B in 2 min and kept for 1.5 min to remove lipophilic components, finally returned to initial conditions over 5 min. Further 1.5 min was allowed for re-equilibration before the next injection. The separation of $3,3'$-T_2/$3,5$-T_2 and T_3/rT_3 was based on the differences of their log Kow (octanol/water partition coefficient) values because they are isobaric compounds and show the same m/z fragmentation. The entire flow was directed into the MS system. Other parameters were as follows: column temperature, 40 °C; flow rate, 5 μL/min.

The electrospray positive ionization mode was adopted for the measurement. The capillary extraction voltage was 2.6 kV. The microchannel plate (MCP) detector potential was 2100 V. The cone voltage was 35 V. We used high purity nitrogen as desolvation gas and auxiliary gas. Argon was used as collision gas. The temperature and flow rate of the desolvation gas were 120°C and 200 L/h, respectively. The cone gas flow rate was 50 L/h. The source temperature was 100°C. The collision energy was 6 eV. We collected the data in the full-scan mode over the range 100–1000 m/z. The scan time and inter scan time were 1.5s and 0.1s, respectively.

A QuanLynx Application Manager software (Waters-Micromass, Manchester, UK) was adopted to acquire and process data. An isotope-dilution method was adopted for the quantification of the analytes. $^{13}C_6$-$3,3'$-T_2 was used as the quantification standard for $3,3'$-T_2, T_1 and 3-T_1AM because isotopically labeled analogues for these compounds were not commercially available.

2.2.5 Method validation

We made calibration curves for all THs in neat solvents and in fortified human placenta.

Calibration standards ranging from 0.5 to 150 pg/µL, with 10 pg/µL of quantification standards were prepared in ACN: H_2O (2:8, v/v) containing 0.1% formic acid. We obtained the matrix-matched calibration curves by spiking THs at different concentrations (0, 2, 5, 10, 20, 50, 100, 150 pg/µL) in the extract, corresponding to a final concentration of 0–18 ng/g in placenta sample.

Matrix effects were evaluated by calculating the percentage of signal enhancement/suppression, as shown in Eq 2.1:

$$\text{Matrix effect} = (A / B - 1) \times 100 \qquad\qquad 2.1$$

where A and B are the slopes of the matrix-matched calibration curve and calibration curve in neat solvent, respectively.

We assessed the accuracy of the method by spike-recovery experiment at different spike levels (0.6, 6 and 12 ng/g). Precision was assessed by intra-day variation and inter-day variation, both were calculated as the %CV of the concentrations of six samples spiked at 0.6, 6 and 12 ng/g. The determination of inter-day CV was carried out over two weeks and included freeze-thaw cycles.

We also calculated the overall recovery for all target analytes. This parameter was defined by comparing the slope of the standard addition curve (C) (spiked before extraction with concentrations of 0, 0.24, 0.6, 1.2, 2.4, 6, 12 and18 ng/g) to the slope of the matrix-matched calibration curve (A), as shown in Eq 2.2:

$$\text{Overall recovery} = C / A \times 100\% \qquad\qquad 2.2$$

ILOD and ILOQ, given as the absolute amount injected on-column, were defined as the lowest concentrations of the analytes in neat solution that produced a signal-to-noise ratio (S/N) of at least 3 (S/N $\geq$ 3) and 10 (S/N $\geq$ 10), respectively. The S/N was calculated using a Peak-to-Peak method on the QuanLynx Application Manager software, in which the noise refers to the difference between the maximum intensity and minimum intensity in the noise region. A time period (> 1 min) after the signal peak was chosen as the signal region (shown in Fig. 2.3). For the calculation of MLOD and MLOQ, fortification of T_1, 3-T_1AM, and 3,5-T_2 in eight blank samples were performed at very low concentration. Three times the SD of the concentrations provides MDL while 10 times the SD provides the MQL. Individual values were

then normalized to the wet placenta sample mass used for extraction to yield a final value of ng/g fresh weight (fw).
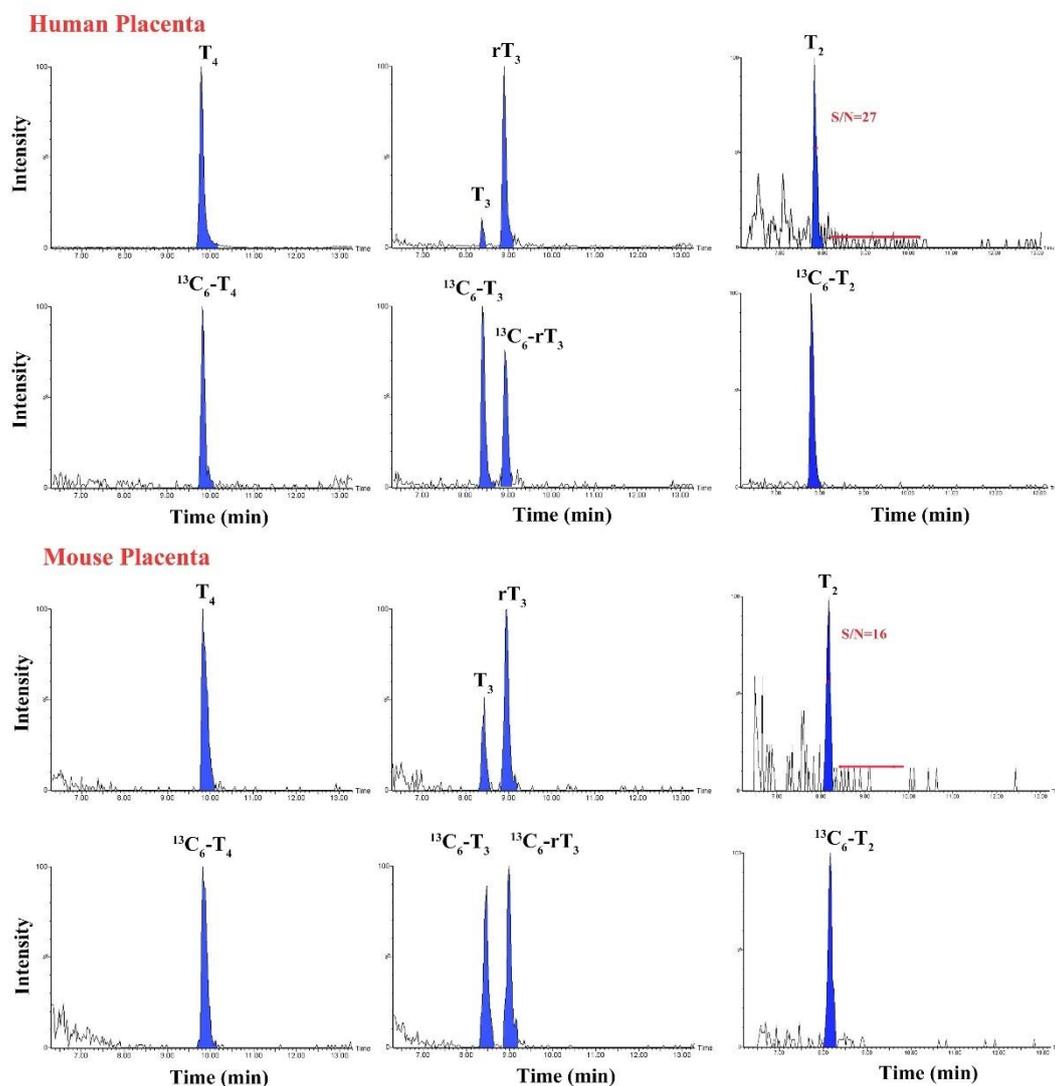
Fig. 2.3 Representative EIC chromatograms of THs detected in human and mouse placenta.

To assess the potential deiodination of the THs, we conducted a "conversion" experiment by spiking $^{13}C_6$-T_4, $^{13}C_6$-T_3 and $^{13}C_6$-rT_3 (10 ng/g of placenta) individually before extraction. No peaks of the metabolites were found in any of the samples analyzed (*i.e.* $^{13}C_6$-T_3, $^{13}C_6$-rT_3, and $^{13}C_6$-3,3'-T_2 in the sample spiked with $^{13}C_6$-T_4) (data not shown). These results confirmed that no conversion of T_4, T_3 or rT_3 happened during the analytical process.

2.2.6 Quality assurance and quality control

Instrumental quality control included regular injection of solvent blanks and standard

solutions. The analytes identification was based on the retention times compared with quantification standards (not allowed to deviate more than 1.0%) and *m/z* ratios of the selected ions. The maximum allowed analyte mass error employed in this study was 0.3 Da. Moreover, we monitored the recovery of $^{13}C_6$-T_4 quantification standard by spiking 0.6 ng of $^{13}C_{12}$-T_4 to each sample before injection. Compared with analytical standards in neat solution, samples that have high deviation (> 40%) on the peak area of quantification standards or lower recovery of recovery standard (< 40%) were discarded and reanalyzed.

2.3 Results and discussion

2.3.1 Chromatography and mass spectrometry

Seven THs (T_4, T_3, rT_3, 3,3'-T_2, 3,5-T_2, T_1 and 3-T_1AM) were targeted during the development and validation of this method, although only T_4, T_3 and rT_3 were found in human placenta previously [42, 43]. Method development started with the optimization of the Q-TOF detection. The full scan mode showed better results than the MS/MS mode (Table 2.1).

The positive mode of the electrospray ionization (ESI (+)) was employed because previous studies observed greater sensitivity for THs in ESI (+) compared with ESI (-) mode [37]. The parameters in ESI (+) mode were optimized by injecting TH standard solutions individually. The optimized parameters are shown in Table 2.2.

To acquire accurate mass measurements, a 2 μg/mL leucine enkephalin ([M+H]$^+$, m/z 556.2771) solution was directed into the MS source at 5 μL/min using a syringe pump system.

To optimize the parameters for data processing, we processed a set of data with different mass extraction windows (± 0.1, 0.2, 0.3, 0.4, 0.5, 0.7 Da). The best signal intensity was obtained when ± 0.3 Da was adopted as the mass window whereas better S/N ratios but lower intensity were observed using smaller mass windows (data not shown). Therefore, all the data in this study was processed using ± 0.3 Da as the mass window.

Table 2.1 Optimized MS parameters for the analytes in this study.

Analytes	Extracted ion (m/z)	Cone voltage (V)	Scan time (s)	Inter scan time (s)	Collision energy (V)
T_4	777.6136, 731.6235, 633.7390	40	1.2	0.1	25
ML-T_4[a]	783.6556, 737.5961, 639.7341	40	1.2	0.1	25
T_3	651.7252, 605.7281, 507.7693	35	1.2	0.1	24
ML-T_3[a]	657.6787, 611.7717, 513.8126	35	1.2	0.1	24
rT_3	651.7252, 605.7081, 507.7142	35	1.2	0.1	24
ML-rT_3[a]	657.6787, 611.7919, 513.7924	35	1.2	0.1	24
3,3'-T_2	525.8195, 479.8035, 381.8938	35	1.2	0.1	22
3,5-T_2	525.8476, 479.7946, 382.8495	35	1.2	0.1	21
ML-3,3'-T_2[a]	531.8093, 485.8396, 387.9027	35	1.2	0.1	22
T_1	399.9073, 353.9423, 256.0827	25	1.0	0.1	19
3-T_1AM	356.9440, 338.9448, 212.0749	25	1.2	0.1	15

[a]Mass labeled internal standards.

Table 2.2 Optimized MS parameters in ESI (+) mode[a]

MS Parameters	Value
MCP potential (V)	2100
Capillary extraction voltage (kV)	2.6
Cone voltage (V)	35
Desolvation gas temperature (°C)	120
Desolvation gas (L h^{-1})	200
Cone gas (L h^{-1})	50
Scan range (m/z)	100–1000
Scan time (s)	1.5
Inter scan time (s)	0.1

[a]The MS parameters were optimized by injection of standard solutions of the THs (10 pg/μL in water/acetonitrile 80:20 with 0.1% (v/v) formic acid).

2.3.2 Sample preparation

Disrupting the protein-THs binding is essential for determining the total content of THs in placenta because the majority of THs in blood or tissues are bound to proteins. Besides, proteins and phospholipids existing in tissues with significant concentrations can cause matrix effect, which is a great challenge in sample cleanup and method validation [167].

In this study, we initially employed a solid/liquid extraction and a liquid/liquid extraction recommended by Gordon *et* al. [168], which has been proven to be able to eliminate proteins and lipids significantly. The calcium salts of the gangliosides and acidic lipids have very low water-solubility. Thus, adding calcium ion could depress gangliosides ionization, leading most of the them remain in chloroform [168]. Briefly, a solution of $CHCl_3$: MeOH (2:1, v/v) was used to extract the compounds from the tissue homogenate. Thereafter, 0.05% $CaCl_2$ was added to extract the analytes into the aqueous phase, and the pooled aqueous phases were further processed.

We used a HybridSPE®-PL cartridge to further eliminate proteins and phospholipids. Formic acid was added to reach a concentration of 3% in the extract. After vortex, the mixture was loaded onto the HybridSPE®-PL cartridge, and the elute was injected into the LC-MS directly. However, after several injections, the signal sensitivity reduced significantly, probably because of the other endogenous compounds (e.g. salts, etc.) [38]. Alternatively, we investigated several SPE cartridges including weak anion-exchangers (Bond Elut Plexa PAX), weak cation-exchangers (Bond Elut Plexa PCX), and mixed-mode polymeric materials (Bond Elut Plexa and SampliQ OPT). Matrix effect and recoveries were used for the assessment of these cartridges. The weak-cation exchanger PCX cartridge displayed the best performance on the clean-up of the analytes (Table 2.3). Therefore, we selected this cartridge for further optimization.

The intention of using antioxidants was to suppress potential free radical oxidation by lipid hydroperoxides and/or metal induced oxidation during the sample preparation process [169]. PTU is an inhibitor of human thyroid iodide peroxidase [170] and was used as an antioxidant for the TH analysis. However, PTU produced a peak at the same retention time as 3-T_1AM and affected the analysis of 3-T_1AM significantly. Alternatively, an antioxidant

solution (10 mg/mL citric acid, ascorbic acid and dithiothreitol) was added before extraction. However, some acid-soluble interferences were extracted and therefore induced serious matrix effects. Using liquid/liquid extraction or SPE was not able to separate these compounds. Therefore, we added the antioxidants after the liquid/liquid extraction to avoid the introduction of these interferences.

Table 2.3 Comparison of the performance of different SPE cartridges used in this study. The recoveries were obtained using 50 ng/mL standard solution.

Recovery (%)	PCX	PAX	Bond Elut Plexa	Sampli Q OPT
T_4	119 ± 1	105 ± 8	143 ± 18	131 ± 3
T_3	109 ± 3	119 ± 12	125 ± 8	130 ± 4
rT_3	110 ± 12	111 ± 11	131 ± 13	140 ± 7
$3,3'-T_2$	110 ± 5	111 ± 3	122 ± 7	118 ± 2
$3,5-T_2$	113 ± 7	96.4 ± 5.9	136 ± 10	143 ± 5
$3-T_1AM$	81.1 ± 1.1	31.8 ± 1.2	3.46 ± 1.98	60.2 ± 4.3
T_1	108 ± 15	88.1 ± 5.1	137 ± 10	183 ± 19

Decreasing the essential sample amount is important for THs analysis in specific placenta regions. Leonetti *et* al. employed 200 mg placenta samples in their LC-QqQ-MS analysis [42]. We also tried to decrease the sample needed in this study. After optimization, 100 mg placenta sample is enough for the quantification of T_4, T_3 and rT_3, while 500 mg sample is essential for the quantification of $3,3'-T_2$.

2.3.3 Method validation

(1) Linearity

We assessed the linearity of the calibration curve using a ten-point calibration curve of the analytes in solvent (0–150 pg/µL), spiked placenta (0–18 pg/g), as well as in extract (0–150 pg/µL). We chose the number of levels to achieve the optimal concentration range for each compound because of the large differences in endogenous levels. The calibration standard was determined in triplicate. Calibration curves were constructed by plotting the ratio between analyte peak area and quantification standard peak area, against the concentration of the THs

injected. The linearity was obtained for all the THs in neat solvents with R^2 values between 0.995 and 0.998.

(2) Precision

We used intra-day variation and inter-day variation to evaluate the precision of this method. As presented in Table 2.4, the intra-day CVs and inter-day CVs for all analytes ranged from 0.5% to 10.3% and 1.19% to 8.88%, indicating that the optimized approach was of good precision for the quantification of these analytes.

(3) Accuracy

We assessed the accuracy of this method by spike-recovery studies. Average recoveries of each compound at different spike levels (0.6, 6 and 12 ng/g) were measured by conducting the test in triplicate. As shown in Table 2.4, the recoveries for all THs except for T_1 and 3-T_1AM were between 81.0% and 112%, and the CV of triplicate analyses was 0.5%–6.2%. These results prove excellent accuracy of the optimized method for determining T_4, T_3, rT_3, 3,3'-T_2, and 3,5-T_2. However, the recoveries of T_1 and 3-T_1AM at 0.6 ng/g spike level were 85.2% and 90.8%, respectively, with relatively high CV values (8.8% and 19.8%), suggesting lower precisions of these less iodinated analytes. This result agrees well with a previous study, which recommended to use isotope-labeled analogues for these analytes [29]. However, these standards are currently not commercially available.

Additionally, the accuracy was verified by overall recovery, which indicates the recovery of each compound in the procedure within the whole working range of concentrations. As presented in Table 2.4, the overall recoveries of T_4, T_3, rT_3, 3,3'-T_2 and 3,5-T_2 were between 60.6% and 84.0%. 3-T_1AM and T_1 presented recoveries lower than 50% probably due to their relatively higher polarities. Although the overall recoveries for some analytes were not close to 100%, they were still considered as acceptable because they were reproducible.

(4) IDLs/MDLs and IQLs/MQLs

IDL and IQL were calculated by analyzing standard solutions that produce a S/N value of at least 3 and 10, respectively. IDLs were 0.25 pg/μL (injected amount 1.3 pg) for T_3, 3,3'-T_2 and 3,5-T_2, 0.5 pg/μL (2.5 pg) for T_4, rT_3, T_1 and 3-T_1AM. IQL values ranged 0.5 pg/μL to 2 pg/μL (2.5–10 pg on-column) (Table 2.4). MDLs and MQLs were measured as 3 or 10 times

the SD of eight blank samples fortified with very low concentrations of T_1, $3\text{-}T_1AM$ and $3,3'\text{-}$ T_2. As shown in Table 2.4, the MDLs were between 0.01 ng/g and 0.2 ng/g, while the MQLs were from 0.04 ng/g to 0.7 ng/g.

Table 2.4 Method evaluation parameters of the developed method for analyzing THs in human placenta. ME, matrix effect; OR, overall recovery.

	T_4	T_3	rT_3	$3,3'\text{-}T_2$	$3,5\text{-}T_2$	T_1	$3\text{-}T_1AM$
ILOD (pg)	2.5	1.3	2.5	1.3	1.3	2.5	2.5
ILOQ (pg)	10	2.5	5.0	2.5	2.5	7.5	3.8
ME (%)	−19.8	−42.3	9.65	−33.7	−29.5	−24.9	−21.9
OR (%)	67.8	77.7	84.0	66.4	60.6	47.5	47.1
Spike-recovery (%), n=3							
0.6 (ng/g)	87.2 ± 2.4	104 ± 5	100 ± 2	102 ± 4	112 ± 0	85.2 ± 8.8	90.8 ± 19.8
6 (ng/g)	103 ± 6	104 ± 3	86.9 ± 6.2	97.2 ± 3.2	94.2 ± 1.0	76.3 ± 4.1	86.7 ± 5.2
12 (ng/g)	105 ± 4	99.1 ± 0.5	81.0 ± 3.2	95.8 ± 3.0	94.6 ± 3.5	77.9 ± 4.7	80.1 ± 5.3
Intra-day Precision (%), n=3							
0.6 (ng/g)	0.96	2.86	2.93	6.80	1.81	10.3	9.80
6 (ng/g)	2.85	2.38	4.19	3.21	1.06	5.36	5.99
12 (ng/g)	3.58	0.52	2.89	3.07	3.72	6.00	6.55
Inter-day Precision (%), n=6							
1.2 (ng/g)	6.38	3.98	1.19	3.90	5.71	8.88	7.06
6 (ng/g)	3.28	2.00	3.21	2.54	3.45	7.41	5.30

(1) Matrix effect

Matrix effect is a well-known phenomenon in LC-MS analysis, particularly in the ESI mode, caused by the suppression or enhancement in the ionization source, and consequently, an increase or decrease in detector responses. The exact mechanisms of matrix effects remain yet unexplained, and various physical and chemical processes are likely responsible [171, 172]. Use of isotope labeled analogs is the best way to compensate for a matrix effect, because the target compounds and the isotope labeled analogs are suppressed or enhanced to the same

extent during ionization. However, the isotope labeled analogs are expensive or sometimes not commercially available.

To investigate the matrix effect, we used the Eq (1). Matrix effect between −20% and 20% is considered as weak ion suppression/enhancement [44]. As shown in Table 2.4. rT_3 presented weak signal enhancement. T_4, $3,3'$-T_2, $3,5$-T_2, 3-T_1AM, and T_1 showed weak to moderate ion suppression (ranging from −33.3% to −19.8%), while a significant ion suppression was observed for T_3, probably because T_3 is more sensitive to the interferences in the matrix. Additionally, $3,5$-T_2, T_1 and 3-T_1AM showed similar ion suppression as $3,3'$-T_2, indicating that $^{12}C_6$-$3,3'$-T_2 is a proper internal standard for these analytes.

Phospholipids are considered as the most important interferences in tissues and blood due to their high abundance. Phosphatidylcholines and lysophosphatidylcholines are the most abundant [167]. The Q-TOF-MS method has the advantage in the identification and characterization of the co-eluents by extracting the EIC spectra from the TIC spectrum. As shown in Fig. 2.4, we compared the ion chromatograms of the phospholipids in a placenta sample before and after extraction.
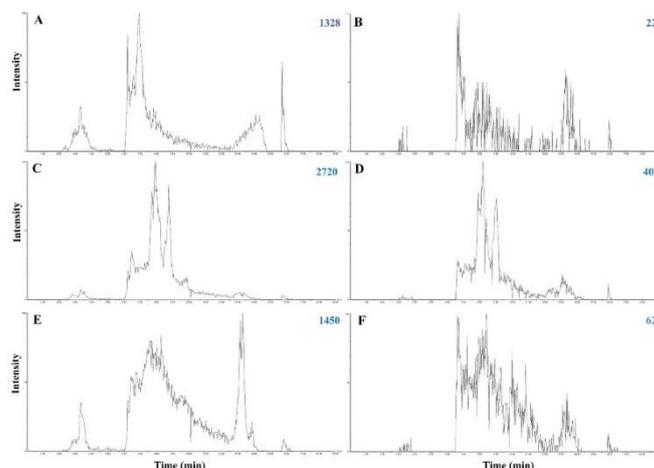
Fig. 2.4 EIC chromatograms of the phospholipids in human placenta sample treated with the optimized procedure. (A) Phosphatidylcholine (*m/z* 758.5094) before extraction; (B) Phosphatidylcholine (*m/z* 758.5094) after extraction; (C) Lysophosphatidylcholine (*m/z* 566.3463) before extraction; (D) Lysophosphatidylcholine (*m/z* 566.3463) after extraction; (E) Lysophosphatidylcholine (*m/z* 496.3398) before extraction; (F) Lysophosphatidylcholine (*m/z* 496.3398) after extraction.

With the optimized procedure, phosphatidylcholines (*m/z* 758.5094) and lysophosphatidylcholine (*m/z* 566.3463) were significantly removed from the matrix. However, lysophosphatidylcholine (*m/z* 496.3398) was still found at a high concentration and was the main contributor for the observed matrix effects.

2.3.4 Application

We further evaluated the optimized method by analyzing human placenta samples, as shown in Table 2.5.

The contents of T_4, T_3 and rT_3 in five human placentas were 22.9–35.0, 0.32–0.46 and 2.86–3.69 ng/g ww, respectively. In 1987, Yoshida and colleagues developed a radio immunoassay (RIA) method for TH measurement in human placenta. T_4, T_3 and rT_3 measured in their study were 18.8, 0.026 and 1.70 ng/g ww, respectively [43]. Later Leonetti *et al*. assessed THs in an epidemiological study using an LC-MS/MS method. The reported concentrations of T_4, T_3 and rT_3 were 11.8–53.6, 0.10–0.84 and 0.73–7.59 ng/g ww, respectively [42], which agree well with the results reported here.

We also applied this method to identify and quantify THs in mouse placenta. As shown in Table 2.6, the concentration of T_4 (2.05–3.51 ng/g ww) was lower than in human placenta, while T_3 (0.37–0.62 ng/g ww) and rT_3 (0.96–1.3 ng/g ww) were at the same level as those in human placenta.

We tracked the presence of 3,3'-T_2 in human and mouse placentas for the first time with concentrations of 0.16–0.26 and 0.07–0.13 ng/g ww (Table 2.5 and 2.6). The 3,3'-T_2 in placenta comes from local metabolism of T_4 because Dio2 and Dio3 are expressed at high level in placenta [173]. Although the exact physiological function of 3,3'-T_2 remains unclear, it has been reported that 3,3'-T_2 regulates cellular respiration through a nuclear-independent pathway [15].

This method was applied to quantify THs in other biomatrices such as human serum, mouse kidney, mouse liver, mouse placenta, and mouse plasma (Fig. 2.5). T_4, T_3, and rT_3 were quantified in human placenta, human serum, mouse kidney, mouse placenta, while T_4 and T_3 were measured in mouse liver and mouse plasma. These results proved that the optimized method is of high sensitivity and selectivity.

Table 2.5 Concentrations of THs determined in human placenta, and comparison with those reported previously.

Tissue	T_4 (ng/g)	T_3 (ng/g)	rT_3 (ng/g)	3,3'-T_2 (ng/g)	3,5-T_2 (ng/g)	3-T_1AM (ng/g)	T_1 (ng/g)	Method	Ref
Sample 1	28.5 ± 2.2	0.43 ± 0.07	3.08 ± 0.69	0.26 ± 0.02	< MDL	< MDL	< MDL	LC-QTOF-MS	This study
Sample 2	30.5 ± 0.4	0.32 ± 0.02	3.65 ± 0.06	0.25 ± 0.01	< MDL	< MDL	< MDL	LC-QTOF-MS	This study
Sample 3	22.9 ± 0.9	0.43 ± 0.01	2.86 ± 0.06	0.16 ± 0.01	< MDL	< MDL	< MDL	LC-QTOF-MS	This study
Sample 4	30.7 ± 0.5	0.37 ± 0.05	3.41 ± 0.42	0.17 ± 0.01	< MDL	< MDL	< MDL	LC-QTOF-MS	This study
Sample 5	35.0 ± 1.1	0.46 ± 0.05	3.69 ± 0.14	0.21 ± 0.03	< MDL	< MDL	< MDL	LC-QTOF-MS	This study
Human placenta	28.1	0.37	2.64					LC-MS/MS	[42]
	(11.8–53.6)	(0.10–0.84)	(0.73–7.59)						
Human placenta	18.8 ± 5.9	0.03 ± 0.01	1.70 ± 0.49					RIA	[43]

Table 2.6 Concentrations of THs in mouse placenta analyzed in this study.

Sample	T_4 (ng/g)	T_3 (ng/g)	rT_3 (ng/g)	3,3'-T_2 (ng/g)	3,5-T_2 (ng/g)	3-T_1AM (ng/g)	T_1 (ng/g)
1	2.67 ± 0.46	0.62 ± 0.05	1.02 ± 0.22	0.12 ± 0.01	< MDL	< MDL	< MDL
2	2.41 ± 0.15	0.52 ± 0.05	1.10 ± 0.05	0.10 ± 0.02	< MDL	< MDL	< MDL
3	2.05 ± 0.01	0.39 ± 0.02	0.96 ± 0.05	0.13 ± 0.01	< MDL	< MDL	< MDL
4	3.51 ± 0.31	0.58 ± 0.03	1.26 ± 0.05	0.08 ± 0.01	< MDL	< MDL	< MDL
5	2.17 ± 0.22	0.37 ± 0.05	1.00 ± 0.05	0.07 ± 0.02	< MDL	< MDL	< MDL
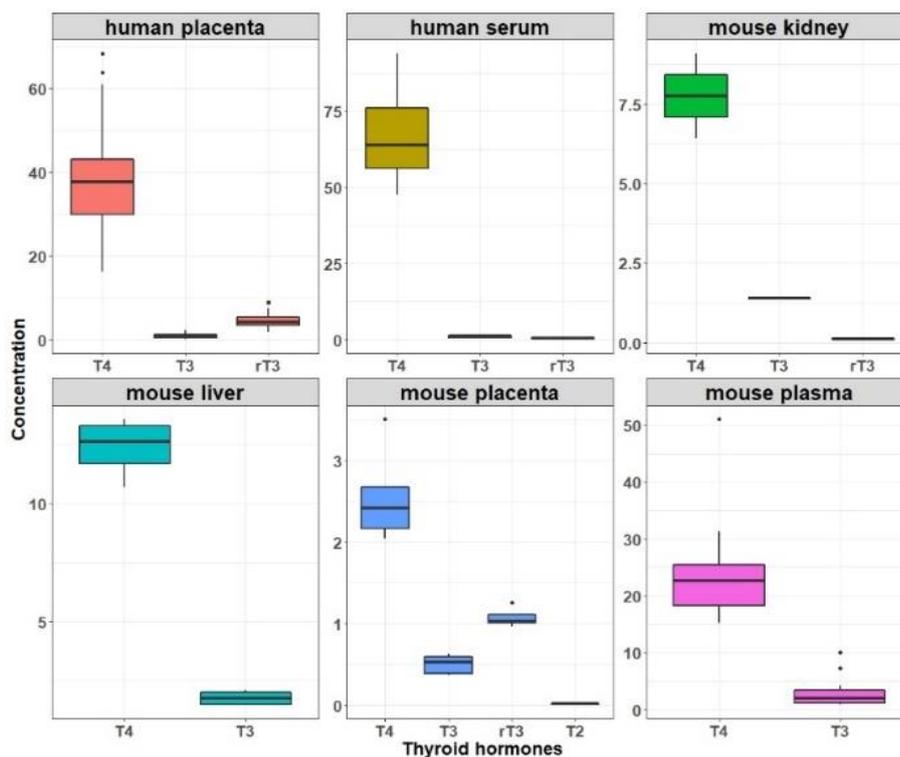
Fig. 2.5 Quantification of THs in various biomatrices using the method with minor modifications. Concentrations are shown in ng/g in tissues and ng/mL in serum/plasma.

2.4 Conclusion

We obtained good validation parameters, including accuracy, linearity, precision, IDLs/IQLs and MDLs/MQLs, suggesting the suitability of the optimized sample preparation procedure for TH analysis in placenta. Applying the optimized method to human placenta and mouse tissues, we observed a good agreement of our method with a previous LC-MS/MS method. The detection of 3,3'-T$_2$ in human/mouse placenta proves that our method is of high sensitivity and selectivity in the analysis of THs. Thus, this method provides a useful tool for research on prenatal TH homeostasis and environmental effects on thyroid function.

Chapter 3 Association between *in utero* persistent organic pollutant exposure and placental thyroid hormones

Abstract

Exposure to POPs during pregnancy can result in thyroid function disorder, leading to concerns about their impact on fetal and neonatal development. In this chapter, we investigated the associations between placental levels of various POPs and THs. In the Danish EXPORED study initially established for assessing congenital cryptorchidism, 58 placenta samples were collected from mothers of boys born with (28) and without (30) cryptorchidism. The concentrations of PBDEs, PCBs, PCDD/Fs, OTCs, OCPs, T_4, T_3, and rT_3 were measured. The associations between placental THs and various POPs were analyzed using multiple linear regression. Five PBDEs, 35 PCBs, 14 PCDD/Fs, 3 OTCs, 25 OCPs, T_4, T_3, and rT_3 were measured. No correlation between THs and the odds of cryptorchidism was found. Several POPs were significantly associated with THs: a) T_4 was inversely associated with BDE-99, -100, ΣPBDE, and 2,3,7,8-TCDD, and positively associated with 1,2,3,4,6,7,8-HpCDF; b) T_3 was positively associated with 2,3,7,8-TCDF and 1,2,3,7,8-PeCDF; c) rT_3 was positively associated with PCB-81, 1,2,3,7,8-PeCDF, and 2,3,4,6,7,8-HxCDF, and inversely associated with tributyltin (TBT), ΣOTC, and methoxychlor (MOC). These results revealed that POP exposures were associated with TH levels in placenta, a possible mechanism for the impacts of POP exposures on children's growth and development.

Author contribution:

Zhong-Min Li was involved in the study design, data analysis, data interpretation, and

manuscript preparation. David Hernadez-Moreno performed the sample measurement and data acquisition. Katharina Maria Main, Niels Erik Skakkebæk, Hannu Kiviranta, Jorma Toppari, Ulla Feldt-Rasmussen, and Heqing Shen were involved in study design, data interpretation, and manuscript review. Karl-Werner Schramm and Meri De Angelis were involved in the study design, sample collection, data interpretation, and manuscript review.

3.1 Introduction

This chapter introduces an epidemiological assessment of the associations between placental levels of various POPs and THs. In a prospective Danish study initially established for assessing congenital cryptorchidism, 58 placenta samples were collected from mothers of boys born with (28) and without (30) cryptorchidism. The concentrations of PBDEs, PCBs, PCDD/Fs, OTCs, OCPs, T_4, T_3, and rT_3 were measured. The associations between placental THs and various POPs were analyzed using multiple linear regression and multipollutant approaches.

3.2 Materials and methods

3.2.1 Study population

The placenta samples were obtained from EXPORED study, which is a joint prospective, longitudinal birth cohort study performed from 1997 to 2001 at the National University Hospital (Rigshospitalet, Hvidovre Hospital), Copenhagen, Denmark. The standardized recruitment strategy, inclusion criteria, participation rate and clinical examination techniques have been reported earlier with details [165, 166]. Placentas were collected at birth by the midwives and kept frozen in polyethylene bags at -20 °C. Upon analysis, placentas were defrosted, mechanically homogenized and aliquoted into 20-mL glass tubes. Fifty-eight placenta samples were derived from mothers of healthy boys (n=30) and mothers of boys with cryptorchidism (n=28) at birth in a nested case-control design. The number of included placenta samples was determined by funding. The demographical variables of both groups are shown in Table 3.1.

This study followed the Helsinki II declaration (World Medical Association 2004). The Danish ethics committee (KF01-030/97) and the Danish Data Protection Agency (1997-1200-074) approved the study. Informed written consent was obtained from the parents of each boy.

Table 3.1 Geometric means and ranges of the demographic characteristics among cryptorchidism cases and controls.

	Cryptorchidism (n=28)	Control (n=30)	p-value[*]
Age (years)	30.6 (25.8 to 39.2)	30.3 (21.0 to 36.3)	0.77
BMI (kg m^{-2}) [a]	22.9 (17.9 to 36.1)	22.4 (18.3 to 37.6)	0.59
Smoking			0.58
Yes	8	11	
No	20	19	
Mode of delivery			0.15
Vaginal delivery	23	26	
Vacuum extraction	3	0	
Cesarean section	2	4	
Parity			0.92
1	27	18	
2	1	9	
≥3	0	3	
Gestational age (days)	277 (248 to 294)	282 (253 to 298)	0.06
Birth weight (kg)	3.49 (2.21 to 4.71)	3.58 (2.46 to 4.55)	0.49
Birth length (cm)	52.3 (45.0 to 60.0)	52.2 (48.0 to 56.0)	0.86

*Difference between cryptorchid boys and controls were analyzed by Mann-Whitney U-test.

3.2.2 Data collection

We determined the total concentrations of T_4, T_3 and rT_3 in placenta samples. Detailed information about the hormones, sample preparation, extraction procedure, analytical method, reagents, and instrumentation can be found in Chapter 2 and [37].

(1) Determination of PBDEs, PCBs and PCDD/Fs

The method adopted for the determination of PBDEs, PCBs, and PCDD/Fs in placenta has been described in detail previously [174, 175]. In brief, the whole placenta was homogenized in a mixer, and 75 g of the homogenate was lyophilized. After pulverization in a mortar, dichloromethane and cyclohexane (1:1, v/v) were added to form a slurry. ^{13}C-labeled quantification standards were spiked. Afterwards, the slurry was cleaned up by acid hydrolysis and diethyl ether extraction. Finally, these compounds were quantified using gas chromatography/high resolution mass spectrometry.

(2) Determination of OCPs

A detailed description of the method for the quantification of OCPs in human placenta has been reported by Shen *et* al. [144, 166, 176, 177]. Briefly, 10 g of placenta homogenate was extracted with 250 mL of a mixture of acetone and *n*-hexane (2:1, v/v). The extract was collected in a flask weighed in advance and evaporated using a rotary vacuum evaporator at 45 °C. Afterwards, the flask was placed into an exicator until stable weight was achieved for gravimetric fat determination. The residual was then dissolved in toluene and extracted using permeation-chromatography and sandwich cartridge cleanup procedure. Finally, the compounds were quantified using a gas chromatography/high resolution mass spectrometry.

(3) Determination of OTCs

The method for OTC analysis has been reported in detail previously [178]. In brief, 0.25 g of lyophilized placenta homogenate was digested with tetramethyl ammonium hydroxide and methanol, buffered to pH4 with sodium acetate-acetic acid. The OTCs were ethylated with sodium tetraethylborate, extracted to hexane, and purified with alumina column. Finally, OTCs were determined using gas chromatography/mass spectrometry.

(4) Determination of lipid content

The concentrations of PBDEs, PCBs, PCDD/Fs, and OCPs were expressed on a lipid basis. The method for lipid content measurement in the placenta samples have been explained previously [144, 176, 177].

In addition, we included the World Health Organization toxic equivalent (WHO-TEQ) values of PCDD/Fs and PCBs. This parameter was developed to measure the combined toxicity

of dioxin and dioxin-like compounds through the toxic equivalency factors (TEFs), which assesses the toxicity of each congener relative to 2,3,7,8-TCDD [179].

3.2.3 Statistics

We determined the potential effects of POPs on thyroid status for congeners with a detection frequency > 50%, except for dibutyltin (DBT), tributyltin (TBT), and triphenyltin (TPhT), which were detected in 38%, 41% and 34% of the samples, respectively. We included OTCs regardless of detection frequency because of the growing concerns about environmental effects as well as a lack of studies on the TH effects of these chemicals. Concentrations below LOQs were replaced by the LOQ divided by the square root of 2. POP concentrations were heavily right-skewed and were $\log_{10}$-transformed to reduce the influence of outliers. T_4, T_3 and rT_3 were normally distributed and not transformed. Normality was confirmed using Kolmogorov-Smirnov test. We used Spearman's rank correlations to evaluate the interrelationship of POP congeners and analysis of variance (ANOVA) to examine associations between demographic characteristics and POP concentrations. Potential confounders considered for inclusion in the models were parameters known to influence THs, i.e., maternal age, gestational age, parity (nulliparous vs. one or more live birth), maternal pre-pregnant body mass index (BMI), smoking during pregnancy (yes vs. no), mode of delivery (three categories: vaginal delivery, vacuum extraction, and cesarean section), birth weight, and birth length. Final models included variables that were loosely associated with the THs ($p < 0.20$) in bivariate analyses.

Adjusted and unadjusted logistic regressions were used to compare placental TH concentrations in cases with cryptorchidism and controls. As no differences were observed, cases and controls were pooled for analysis of the association between POPs and THs by multiple linear regression for individual POP congeners and the sums.

For multipollutant assessment, we used partial least squares (PLS) regressions to estimate the impact of all POPs and covariates simultaneously on placental THs. For data reduction and to increase the model predictive ability, only variables with variable importance to projection (VIP) values > 0.4 were included in the final model. For highly correlated covariates sensitive to pregnancy-related changes, principal component analysis (PCA) was performed. The score

of each woman on the first principal component was included as a common pregnancy vector in multiple linear regression models to avoid collinearity issues while adjusting for these factors. To minimize the number of contaminants to be included in linear regression models, hierarchical clustering analysis of POPs based on correlations (method: complete linkage) was performed, and groupings according to clusters were subsequently performed by simple addition of POP concentrations.

All statistical analyses were conducted using SAS (version 9.4; SAS Institute Inc., Cary, NC) and R (version 3.4.2; R Foundation for Statistical Computing, Vienna, Austria). A p-value < 0.05 was considered significant, and $p < 0.10$ was considered as a tendency of association.

3.3 Results

Table 3.2 summarizes the characteristics of all the participating women and newborns. The mean ($\pm$ SD) age of women was 30.4 ± 3.5 years. Among them, 19 (32.8%) were > 30 years of age; forty-seven (81%) had a BMI value of < 25; thirty-nine (67.2%) did not smoke; forty-nine (84.5%) had a normal delivery; thirty-five (60.3%) were nullipara; fifty-four (93.1%) babies were delivered at over 37 weeks of gestation (full term). The demographical parameters did not differ significantly between the cryptorchid and control groups except for gestational age (p=0.06) (Table 3.1).

Table 3.2 Correlations between demographic characteristics and TH placental concentrations using t-test and analysis of variance (ANOVA).

Characteristics	n	%	Measures of THs in placenta (mean (SD))		
			T_4 (ng/g fw)	T_3 (ng/g fw)	rT_3 (ng/g fw)
Mean (SD)	58	100	37.6 (10.9)	0.85 (0.50)	4.57 (1.63)
Median (minimum, maximum)	58	100	37.7 (16.3, 68.3)	0.69 (0, 2.34)	4.21 (1.82, 9.03)
Maternal characteristics					
Age (years)					
< 30	39	67.2	35.5 (10.4)	0.88 (0.51)	4.65 (1.72)

≥30	19	32.8	42.1 (11.0)	0.79 (0.49)	4.41 (1.47)
BMI (kg/m^2)					
< 25	47	81.0	37.4 (10.9)	0.86 (0.52)	4.72 (1.68)
≥25	10	17.2	38.4 (11.9)	0.73 (0.38)	4.05 (1.28)
Smoking					
Yes	19	32.8	42.1 (11.0)*	0.79 (0.49)	4.41 (1.47)
No	39	67.2	35.5 (10.4)	0.88 (0.51)	4.65 (1.72)
Mode of delivery					
Vaginal delivery	49	84.5	37.4 (11.5)	0.83 (0.51)	4.63 (1.61)
Vacuum extraction	3	5.17	43.6 (1.77)	1.14 (0.50)	5.48 (3.03)
Caesarean section	6	10.3	36.7 (7.89)	0.82 (0.47)	3.66 (0.65)
Parity					
1	35	60.3	36.8 (10.6)	0.87 (0.51)	4.62 (1.65)
2	18	31.0	37.9 (10.3)	0.84 (0.55)	4.45 (1.80)
3	5	8.62	42.4 (16.4)	0.75 (0.23)	4.71 (1.00)
Gestational age (days)					
< 268	7	12.1	31.0 (7.3)*	0.74 (0.46)	3.92 (0.48)
268–278	12	20.7	33.1 (10.5)	0.99 (0.59)	4.29 (1.82)
278–288	25	43.1	38.7 (9.5)	0.84 (0.49)	4.80 (1.82)
≥288	14	24.1	43.0 (12.8)	0.79 (0.48)	4.43 (1.50)
Infant characteristics					
Birth weight (kg)					
< 3	5	8.62	28.6 (5.2)*	0.90 (0.46)	3.69 (0.65)
3–4	43	74.1	37.0 (9.8)	0.83 (0.53)	4.62 (1.65)
≥4	10	17.2	44.9 (13.8)	0.90 (0.39)	4.82 (1.86)
Birth length (cm)					
< 50	5	8.62	31.8 (7.6)	0.83 (0.55)	3.76 (0.63)
50–55	43	74.1	37.1 (10.4)	0.86 (0.53)	4.74 (1.71)
≥ 55	10	17.2	42.9 (13.2)	0.83 (0.35)	4.26 (1.57)

As shown in Table 3.2, T_4 was measured in the highest concentration with a mean of 37.6 ng/g fresh weight (fw) (range: 16.3–68.3 ng/g fw), followed by rT_3 with a mean of 4.57 ng/g fw (range: 1.82–9.03 ng/g fw). T_3 was measured in the lowest concentration with a mean of 0.85 ng/g fw (range: 0–2.34 ng/g fw). TH levels were generally similar across age, BMI, parity, mode of delivery, and birth length of the infants. T_4 was correlated with gestational age ($p = 0.01$), birth weight ($p = 0.06$), and smoking ($p = 0.03$). However, T_3 and rT_3 were not correlated with any of the studied characteristics. Placental concentrations of THs did not significantly differ between cryptorchid and control boys (Table 3.3).

Table 3.3 Association between placental TH concentrations and odds of cryptorchidism.

THs (ng/g)	OR[a] (95% CI[b])	
	Unadjusted	Adjusted[c]
T_4	1.05 (1.00 to 1.12)	1.04 (0.98 to 1.11)
T_3	0.50 (0.14 to 1.60)	0.53 (0.15 to 1.73)
rT_3	1.31 (0.93 to 1.94)	1.35 (0.94 to 2.05)

[a]Odds ratio.
[b]95% confidence intervals.
[c]Models adjusted for maternal smoking, gestational age and infant birth weight.

The concentrations of a total of 82 POPs and their sums, as well as the WHO-TEQ values of PCDD/Fs and PCBs in placenta are shown in Table 3.4. The limit of quantification (LOQ) values were 0.004–0.14 ng/g lipid for PBDEs [174], 0.17 pg/g lipid for non-*ortho*-PCBs, 0.01 ng/g lipid for mono- and di-*ortho*-PCBs, 0.12–0.25 pg/g lipid for tetra to hepta chlorinated PCDD/Fs, 1.2 pg/g lipid for octa chlorinated PCDD/Fs [180], 0.1 ng/g fw for DBT, 0.02 ng/g fw for TBT and TPhT [178]. The limit of detection (LOD) values of the OCPs ranged from 0.01 ng/g lipid to 1.24 ng/g lipid [166].

As shown in Table 3.5, Table 3.6 and Fig. 3.1. T_4 was inversely significantly associated with the sum of 5 PBDEs (β=-19.0; 95% CI: -35.7, -2.37; $p < 0.026$) as well as with BDE-99 (β=-20.2; 95% CI: -35.2, -5.29; $p < 0.009$), BDE-100 (β=-13.5; 95% CI: -26.8, -0.22; $p < 0.047$)

with a tendency for BDE-47 (β=-14.1; 95% CI: -28.5, 0.24; $p < 0.054$). T_3 and rT_3 were not significantly associated with any of the PBDE congeners.

T_4 showed negative tendencies with PCB 99 (β=-15.9; 95% CI: -33.4, 1.65; $p < 0.075$), PCB-118 (β=-13.4; 95% CI: -29.0, 2.27; $p < 0.092$) and PCB-167 (β=-11.7; 95% CI: -25.7, 2.29; $p < 0.099$). No statistically significant associations between T_3 and PCB congeners was observed. rT_3 was positively significantly associated with PCB-81 (β=2.55; 95% CI: 0.37, 4.72; $p < 0.023$) with a positive tendency for PCB-101 (β=0.80; 95% CI: -0.06, 1.65; $p < 0.067$) and PCB-183 (β=2.08; 95% CI: -0.35, 4.51; $p < 0.091$).

T_4 was inversely significantly associated with 2,3,7,8-TCDD (β=-18.4; 95% CI: -34.6, -2.25; $p < 0.026$) with a tendency for 1,2,3,7,8-PeCDD (β=-13.4; 95% CI: -28.8, 2.01; $p < 0.087$), while positively and significantly associated with 1,2,3,4,6,7,8-HpCDF (β=14.1; 95% CI: 4.37, 22.8; $p < 0.005$) with a positive tendency for OCDD (β=10.8; 95% CI: -1.94, 23.5; $p < 0.095$). T_3 was positively significantly associated with 2,3,7,8-TCDF (β=1.08; 95% CI: 0.33, 1.83; $p < 0.006$) and 1,2,3,7,8-PeCDF (β=0.73; 95% CI: 0.07, 1.40; $p < 0.032$). rT_3 was positively significantly associated with 1,2,3,7,8-PeCDF (β=2.53; 95% CI: 0.41, 4.64; $p < 0.020$) and 2,3,4,6,7,8-HxCDF (β=3.11; 95% CI: 0.94, 5.27; $p < 0.006$), and although not statistically significant, showed a positive tendency with 1,2,3,4,7,8-HxCDF (β=2.68; 95% CI: -0.47, 5.82; $p < 0.093$), 1,2,3,6,7,8-HxCDF (β=2.66; 95% CI: -0.41, 5.73; $p < 0.088$) and 1,2,3,4,6,7,8-HpCDF (β=1.43; 95% CI: -0.24, 3.10; $p < 0.092$).
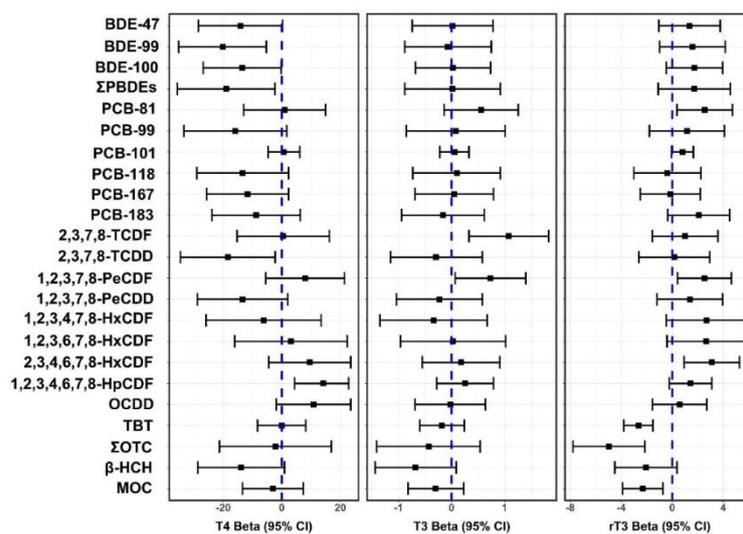
Fig. 3.1 Significant or marginal associations between various POP and TH concentrations in placenta from women participating in the Danish cohort study.

Table 3.4 Placental concentrations of the POP congeners in the Danish Cohort Study. The concentrations of PCDDs, PCDD_WHO-TEQ, non-ortho-PCBs (PCB-81, 77, 126 and 169), and PCB_WHO-TEQ were expressed as pg/g lipid. The concentrations of PBDEs, ortho-PCBs, and OCPs were expressed as ng/g lipid. The concentrations of OTCs were expressed as ng/g fresh weight.

POPs	Detection frequency (%)	GM[b]	95% CI[c]	Min	25th percentile	Median	75th percentile	Max
PBDEs								
BDE-28	100	0.03	0.02–0.05	0.01	0.02	0.03	0.03	0.40
BDE-47	100	0.44	0.37–0.50	0.17	0.32	0.37	0.45	1.46
BDE-99	100	0.21	0.19–0.24	0.08	0.16	0.19	0.25	0.56
BDE-100	100	0.11	0.09–0.13	0.04	0.07	0.10	0.12	0.44
BDE-153	100	0.46	0.40–0.51	0.21	0.30	0.41	0.56	1.29
ΣPBDE	100	1.27	1.13–1.41	0.57	0.97	1.09	1.38	3.24
PCBs								
PCB-18	100	0.11	0.07–0.14	0.02	0.05	0.07	0.11	0.91
PCB-28/31	100	0.59	0.50–0.67	0.21	0.37	0.51	0.67	1.90
PCB-33	98.3	0.06	0.05–0.08	0.01	0.03	0.05	0.07	0.40
PCB-47	100	0.07	0.06–0.07	0.02	0.05	0.06	0.08	0.15
PCB-49	98.3	0.04	0.04–0.05	0.01	0.03	0.04	0.05	0.10
PCB-52	100	0.10	0.09–0.10	0.03	0.08	0.09	0.11	0.21

PCB-60	100	0.11	0.10–0.13	0.05	0.07	0.10	0.14	0.31
PCB-66	100	0.28	0.24–0.31	0.12	0.18	0.24	0.32	0.73
PCB-74	100	1.15	1.05–1.25	0.56	0.91	1.09	1.31	2.75
PCB-77	100	5.10	4.25–5.96	0.24	2.19	5.00	6.77	13.7
PCB-81	96.6	0.55	0.48–0.61	0.12	0.39	0.50	0.65	1.23
PCB-99	100	1.33	1.21–1.46	0.47	1.04	1.29	1.57	3.11
PCB-101	70.7	0.11	0.09–0.14	0.01	0.01	0.09	0.16	0.45
PCB-105	100	0.50	0.44–0.56	0.15	0.33	0.45	0.56	1.04
PCB-110	74.1	0.08	0.06–0.09	0.01	0.02	0.07	0.11	0.43
PCB-114	100	0.12	0.11–0.13	0.05	0.09	0.11	0.13	0.26
PCB-118	100	2.38	2.12–2.64	0.88	1.60	2.13	2.83	4.96
PCB-123	98.3	0.09	0.08–0.10	0.01	0.05	0.08	0.10	0.20
PCB-126	100	10.8	9.57–12.0	2.58	7.17	10.4	13.9	21.3
PCB-128	98.3	0.14	0.12–0.16	0.02	0.09	0.12	0.16	0.46
PCB-138	100	8.65	7.87–9.43	3.30	6.21	8.73	10.5	15.4
PCB-141	75.9	0.04	0.03–0.05	0.01	0.02	0.04	0.06	0.11
PCB-153	100	14.9	13.6–16.3	5.92	10.9	14.7	18.7	26.5
PCB-156	100	1.53	1.37–1.68	0.58	1.12	1.36	1.89	3.44

PCB-157	100	0.24	0.21–0.26	0.08	0.17	0.21	0.31	0.79
PCB-167	100	0.28	0.25–0.31	0.09	0.19	0.26	0.35	0.62
PCB-169	100	7.39	6.60–8.17	2.63	5.20	6.98	9.12	15.0
PCB-170	100	5.12	4.61–5.63	2.17	3.75	4.61	6.52	9.92
PCB-180	100	10.1	9.03–11.8	4.00	7.29	9.12	12.8	20.4
PCB-183	100	1.18	1.06–1.29	0.33	0.86	1.13	1.43	2.31
PCB-187	100	2.21	1.96–2.46	0.63	1.44	2.02	2.78	4.68
PCB-189	100	0.19	0.17–0.21	0.06	0.13	0.17	0.25	0.43
PCB-194	100	1.22	1.08–1.36	0.45	0.84	1.09	1.54	2.60
PCB-206	100	0.26	0.23–0.30	0.07	0.17	0.25	0.35	0.64
PCB-209	100	0.26	0.23–0.29	0.10	0.18	0.24	0.33	0.60
ΣPCB	100	53.4	48.7–58.2	22.1	39.8	53.7	66.8	91.9
PCB_WHO-TEQ	100	2.41	2.19–2.64	0.82	1.72	2.32	2.95	4.26
PCDDs								
2,3,7,8-TCDF	89.7	0.27	0.23–0.30	0.11	0.19	0.25	0.32	0.92
2,3,7,8-TCDD	100	1.44	1.28–1.59	0.56	1.04	1.33	1.63	3.76
1,2,3,7,8-PeCDF	69.0	0.23	0.19–0.26	0.09	0.13	0.20	0.27	0.77

2,3,4,7,8-PeCDF	100	7.88	7.06–8.70	2.88	5.54	7.32	9.73	15.9
1,2,3,7,8-PeCDD	100	5.35	4.69–6.00	2.20	3.85	4.97	6.23	16.4
1,2,3,4,7,8-HxCDF	100	2.08	1.90–2.25	0.99	1.57	1.99	2.47	3.76
1,2,3,6,7,8-HxCDF	100	1.23	1.13–1.33	0.52	0.95	1.18	1.46	2.28
2,3,4,6,7,8-HxCDF	72.4	0.47	0.41–0.53	0.10	0.37	0.46	0.50	1.29
1,2,3,4,7,8-HxCDD	100	3.72	3.25–4.19	1.46	2.52	3.48	4.33	11.8
1,2,3,6,7,8-HxCDD	100	6.73	6.06–7.41	2.65	5.37	6.40	7.59	17.9
1,2,3,7,8,9-HxCDD	100	2.98	2.43–3.54	0.71	1.98	2.51	3.28	15.5
1,2,3,4,6,7,8-HpCDF	96.6	1.57	1.35–1.79	0.16	0.95	1.40	1.99	4.37
1,2,3,4,6,7,8-HpCDD	100	10.5	8.77–12.1	2.16	6.47	9.34	11.6	36.5
OCDD	100	90.2	76.6–104	28.3	61.4	76.4	97.8	302
ΣPCDD	100	134	118–150	45.2	103	124	149	368
PCDD_WHO-TEQ	100	12.6	11.3–13.9	5.05	9.23	12.0	14.4	31.3
OTCs[e]								
DBT	37.9	0.12	0.11–0.13	0.10	0.10	0.10	0.13	0.25
TBT	41.4	0.05	0.03–0.07	0.02	0.02	0.02	0.04	0.32
TPhT	34.5	0.02	0.02–0.03	0.02	0.02	0.02	0.03	0.07

ΣOTC	67.2	0.19	0.17–0.21	0.14	0.14	0.15	0.20	0.60
OCPs								
PeCB	100	0.47	0.40–0.55	0.19	0.29	0.40	0.57	1.95
α-HCH	100	0.83	0.56–1.10	0.04	0.20	0.38	1.12	5.72
(-)-α-HCH	96.6	0.51	0.37–0.65	0.03	0.16	0.29	0.73	2.88
(+)-α-HCH	91.4	0.45	0.31–0.59	0.03	0.12	0.20	0.62	2.89
β-HCH	100	9.18	8.13–10.2	3.41	6.26	8.45	10.8	25.6
γ-HCH	100	0.83	0.74–0.92	0.28	0.58	0.73	1.00	2.04
PCA	100	0.22	0.12–0.31	0.04	0.09	0.13	0.21	2.70
HCB	100	7.89	7.32–8.45	4.26	6.25	7.69	9.13	13.4
OCS	100	0.11	0.09–0.13	0.02	0.07	0.10	0.14	0.34
OXC	100	1.14	0.98–1.30	0.25	0.36	0.63	1.07	2.24
(+)-OXC	91.4	0.72	0.59–0.84	0.12	0.36	0.63	1.07	2.24
(-)-OXC	81.0	0.53	0.43–0.63	0.12	0.19	0.41	0.83	1.54
c-HE	100	0.93	0.83–1.03	0.19	0.64	0.90	1.15	1.77
(+)-HE	98.3	0.60	0.52–0.67	0.04	0.43	0.58	0.78	1.37
(-)-HE	93.1	0.30	0.25–0.35	0.04	0.15	0.28	0.44	0.75
o,p'-DDE	96.6	0.03	0.02–0.03	0.01	0.02	0.03	0.04	0.07

p,p'-DDE	100	49.5	39.3–59.8	9.52	26.8	40.4	56.2	270
END-I	100	2.12	1.82–2.43	0.43	1.07	1.95	2.98	4.90
p,p'-DDD	100	0.87	0.66–1.07	0.19	0.50	0.68	1.01	5.59
o,p'-DDD	98.3	0.07	0.05–0.08	0.01	0.03	0.05	0.09	0.21
Dieldrin	100	2.60	2.23–2.97	0.66	1.59	2.48	3.09	9.53
o,p'-DDT	98.3	0.06	0.05–0.07	0.01	0.03	0.05	0.09	0.16
p,p'-DDT	100	0.59	0.45–0.73	0.19	0.32	0.44	0.71	3.32
MOC	100	0.08	0.06–0.09	0.02	0.04	0.06	0.09	0.36
Mirex	100	0.18	0.14–0.22	0.01	0.08	0.14	0.20	0.94
ΣOCP	100	77.7	66.3–89.2	33.6	50.5	67.8	89.7	303

Abbreviations: OCDD, octachlorodibenzo-*p*-dioxin; DBT, dibutyltin; TBT, tributyltin; TPhT, triphenyltin; PeCB, pentachlorobenzene; HCH, hexachlorocyclohexane; PCA, pentachloroanisole; HCB, hexachlorobenzene; OCS, octachlorostyrene; OXC, oxychlordane; c-HE, *cis*-heptachloroepoxide; END-I, endosulfan-I; MOC, methoxychlor; WHO-TEQ, the World Health Organization toxic equivalent.

[a]Limit of quantification.
[b]Geometric mean.
[c]95% confidence interval.

Table 3.5 Significant or marginal associations between various POPs and TH concentrations in placenta from women participating in the Danish Cohort Study.

POPs[a]	T_4		T_3		rT_3	
	β (95% CI)[b]	R^2	β (95% CI)[b]	R^2	β (95% CI)[b]	R^2
PBDEs						
BDE-47	-14.1 (-28.5 to 0.24)#	0.27	0.02 (-0.74 to 0.78)	0.01	1.36 (-1.04 to 3.77)	0.08
BDE-99	-20.2 (-35.2 to -5.29)**	0.32	-0.07 (-0.88 to 0.75)	0.01	1.59 (-0.99 to 4.16)	0.09
BDE-100	-13.5 (-26.8 to -0.22)*	0.28	0.03 (-0.68 to 0.74)	0.01	1.74 (-0.47 to 3.96)	0.10
ΣPBDE	-19.0 (-35.7 to -2.37)*	0.29	0.02 (-0.88 to 0.92)	0.01	1.72 (-1.10 to 4.55)	0.09
PCBs						
PCB-81	0.92 (-13.0 to 14.9)	0.22	0.56 (-0.14 to 1.26)	0.06	2.55 (0.37 to 4.72)*	0.15
PCB-99	-15.9 (-33.4 to 1.65)#	0.27	0.08 (-0.85 to 1.01)	0.01	1.16 (-1.78 to 4.10)	0.07
PCB-101	0.69 (-4.69 to 6.08)	0.22	0.06 (-0.22 to 0.33)	0.02	0.80 (-0.06 to 1.65)#	0.12
PCB-118	-13.4 (-29.0 to 2.27)#	0.26	0.10 (-0.73 to 0.92)	0.01	-0.38 (-3.01 to 2.25)	0.06
PCB-167	-11.7 (-25.7 to 2.29)#	0.26	0.05 (-0.69 to 0.79)	0.01	-0.14 (-2.50 to 2.21)	0.06
PCB-183	-8.75 (-23.8 to 6.27)	0.24	-0.16 (-0.94 to 0.62)	0.02	2.08 (-0.35 to 4.51)#	0.11
PCDD/Fs						
2,3,7,8-TCDF	0.42 (-15.3 to 16.2)	0.22	1.08 (0.33 to 1.83)**	0.15	1.01 (-1.55 to 3.58)	0.07

2,3,7,8-TCDD	-18.4 (-34.6 to -2.25)*	0.29	-0.29 (-1.15 to 0.58)	0.02	0.16 (-2.62 to 2.94)	0.06
1,2,3,7,8-PeCDF	7.93 (-5.50 to 21.4)	0.24	0.73 (0.07 to 1.40)*	0.10	2.53 (0.41 to 4.64)*	0.15
1,2,3,7,8-PeCDD	-13.4 (-28.8 to 2.01)#	0.26	-0.23 (-1.04 to 0.58)	0.02	1.39 (-1.18 to 3.96)	0.08
1,2,3,4,7,8-HxCDF	-6.23 (-25.9 to 13.4)	0.23	-0.34 (-1.35 to 0.67)	0.02	2.68 (-0.47 to 5.82)#	0.11
1,2,3,6,7,8-HxCDF	3.10 (-16.1 to 22.3)	0.22	0.03 (-0.96 to 1.02)	0.01	2.66 (-0.41 to 5.73)#	0.11
2,3,4,6,7,8-HxCDF	9.51 (-4.46 to 23.5)	0.25	0.18 (-0.55 to 0.91)	0.02	3.11 (0.94 to 5.27)**	0.19
1,2,3,4,6,7,8-HpCDF	14.1 (4.37 to 22.8)**	0.33	0.26 (-0.28 to 0.79)	0.03	1.43 (-0.24 to 3.10)#	0.11
OCDD	10.8 (-1.94 to 23.5)#	0.26	-0.02 (-0.69 to 0.64)	0.01	0.59 (-1.54 to 2.72)	0.07
OTCs						
TBT	-0.07 (-8.33 to 8.19)	0.22	-0.18 (-0.60 to 0.24)	0.03	-2.65 (-3.79 to -1.51)**	0.33
ΣOTC	-2.20 (-21.3 to 16.9)	0.22	-0.43 (-1.41 to 0.54)	0.03	-4.96 (-7.78 to -2.14)**	0.24
OCPs						
β-HCH	-13.9 (-28.7 to 0.90)#	0.27	-0.68 (-1.44 to 0.09)#	0.07	-2.06 (-4.50 to 0.38)#	0.11
MOC	-3.08 (-13.4 to 7.27)	0.23	-0.30 (-0.82 to 0.23)	0.04	-2.30 (-3.88 to -0.72)**	0.19

Abbreviations: OCDD, octachlorodibenzo-p-dioxin; TBT, tributyltin; OCS, octachlorostyrene; β-HCH, β-hexachlorocyclohexane; MOC, methoxychlor.

[a]Placental concentrations of PBDEs, PCBs, PCDD/Fs, OTCs and OCPs were $\log_{10}$-transformed. PBDEs, PCBs, PCDD/Fs and OCPs were expressed on lipid basis, OCTs were expressed on fresh weight basis.

[b]Adjusted for maternal smoking, gestational age, and neonatal birth weight.

*$p < 0.05$. **$p < 0.01$. #$p < 0.10$.

Table 3.6 Non-significant associations between various POPs and TH concentrations in placenta from women participating in the Danish Cohort Study.

POPs[a]	T$_4$		T$_3$		rT$_3$	
	β (95% CI)[b]	R^2	β (95% CI)[b]	R^2	β (95% CI)[b]	R^2
PBDEs						
BDE-28	-9.96 (-22.2 to 2.27)	0.26	-0.05 (-0.69 to 0.59)	0.01	0.12 (-1.94 to 2.17)	0.06
BDE-153	-12.6 (-28.0 to 2.82)	0.26	0.09 (-0.72 to 0.90)	0.01	1.29 (-1.27 to 3.86)	0.08
PCBs						
PCB-18	1.02 (-7.71 to 9.75)	0.22	-0.18 (-0.62 to 0.27)	0.03	-0.63 (-2.05 to 0.79)	0.07
PCB-28/31	-3.33 (-16.8 to 10.2)	0.22	0.08 (-0.61 to 0.78)	0.01	-0.54 (-2.76 to 1.67)	0.07
PCB-33	5.47 (-4.54 to 15.5)	0.24	-0.09 (-0.61 to 0.42)	0.02	-0.39 (-2.05 to 1.26)	0.06
PCB-47	-10.5 (-26.1 to 5.15)	0.25	0.09 (-0.73 to 0.90)	0.01	0.95 (-1.64 to 3.54)	0.07
PCB-49	8.16 (-6.45 to 22.8)	0.24	-0.24 (-0.99 to 0.52)	0.02	0.29 (-2.13 to 2.72)	0.06
PCB-52	14.4 (-3.88 to 32.7)	0.26	-0.04 (-1.00 to 0.92)	0.01	1.34 (-1.70 to 4.38)	0.07
PCB-60	-3.15 (-17.7 to 11.4)	0.22	0.22 (-0.52 to 0.97)	0.02	0.64 (-1.74 to 3.02)	0.07
PCB-66	-4.15 (-19.9 to 11.6)	0.22	0.31 (-0.49 to 1.11)	0.03	0.59 (-1.98 to 3.17)	0.06
PCB-74	-13.4 (-33.5 to 6.75)	0.25	-0.05 (-1.10 to 1.00)	0.01	0.53 (-2.82 to 3.88)	0.06
PCB-77	-2.24 (-9.31 to 4.84)	0.23	-0.10 (-0.46 to 0.27)	0.02	0.41 (-0.75 to 1.57)	0.07

PCB-105	-10.4 (-24.1 to 3.36)	0.25	0.12 (-0.60 to 0.84)	0.02	0.09 (-2.21 to 2.39)	0.06
PCB-110	-0.82 (-7.44 to 5.79)	0.22	-0.04 (-0.38 to 0.30)	0.01	0.78 (-0.28 to 1.84)	0.10
PCB-114	-12.5 (-30.0 to 4.99)	0.25	-0.14 (-1.06 to 0.77)	0.02	-0.50 (-3.42 to 2.42)	0.06
PCB-123	-4.33 (-16.6 to 7.90)	0.23	-0.18 (-0.81 to 0.45)	0.02	0.62 (-1.39 to 2.62)	0.07
PCB-126	-8.34 (-22.6 to 5.87)	0.24	0.41 (-0.32 to 1.14)	0.04	0.64 (-1.71 to 2.99)	0.07
PCB-128	-6.31 (-17.6 to 4.94)	0.24	-0.12 (-0.70 to 0.47)	0.02	1.27 (-0.56 to 3.10)	0.09
PCB-138	-12.6 (-28.9 to 3.70)	0.25	-0.28 (-1.13 to 0.57)	0.02	1.48 (-1.22 to 4.17)	0.08
PCB-141	0.58 (-8.89 to 10.0)	0.22	0.09 (-0.39 to 0.58)	0.02	0.41 (-1.14 to 1.96)	0.07
PCB-153	-12.7 (-29.3 to 3.90)	0.25	-0.17 (-1.04 to 0.70)	0.02	1.27 (-1.49 to 4.03)	0.08
PCB-156	-8.01 (-23.4 to 7.37)	0.24	-0.22 (-1.02 to 0.57)	0.02	0.39 (-2.15 to 2.94)	0.06
PCB-157	-9.84 (-24.9 to 5.26)	0.24	-0.17 (-0.96 to 0.61)	0.02	0.14 (-2.38 to 2.65)	0.06
PCB-169	-6.17 (-21.0 to 8.66)	0.23	-0.10 (-0.87 to 0.67)	0.02	0.91 (-1.52 to 3.35)	0.07
PCB-170	-8.19 (-23.5 to 7.12)	0.24	-0.21 (-1.01 to 0.58)	0.02	1.25 (-1.26 to 3.77)	0.08
PCB-180	-7.36 (-22.4 to 7.63)	0.23	-0.13 (-0.90 to 0.65)	0.02	1.08 (-1.38 to 3.54)	0.07
PCB-187	-8.81 (-22.2 to 4.59)	0.24	0.02 (-0.68 to 0.72)	0.01	0.79 (-1.43 to 3.01)	0.07
PCB-189	-5.89 (-20.0 to 8.26)	0.23	-0.16 (-0.89 to 0.56)	0.02	0.55 (-1.78 to 2.87)	0.06
PCB-194	-4.76 (-18.4 to 8.85)	0.23	-0.03 (-0.73 to 0.67)	0.01	0.85 (-1.38 to 3.07)	0.07
PCB-206	-2.52 (-15.8 to 10.8)	0.22	0.31 (-0.37 to 0.99)	0.03	0.64 (-1.53 to 2.82)	0.07

PCB-209	-6.64 (-20.8 to 7.48)	0.23	0.20 (-0.53 to 0.92)	0.02	0.95 (-1.37 to 3.27)	0.07
ΣPCB	-11.5 (-28.4 to 5.44)	0.25	-0.16 (-1.04 to 0.73)	0.02	1.26 (-1.55 to 4.06)	0.08
PCB_WHO-TEQ	-11.6 (-28.5 to 5.26)	0.25	0.17 (-0.71 to 1.05)	0.01	0.53 (-2.28 to 3.33)	0.06
PCDD/Fs						
2,3,4,7,8-PeCDF	-10.9 (-26.7 to 4.87)	0.25	0.12 (-0.71 to 0.94)	0.02	1.10 (-1.51 to 3.71)	0.07
1,2,3,4,7,8-HxCDD	-6.40 (-21.1 to 8.32)	0.23	-0.50 (-1.24 to 0.25)	0.05	1.12 (-1.29 to 3.53)	0.08
1,2,3,6,7,8-HxCDD	-4.16 (-21.3 to 13.0)	0.22	-0.43 (-1.30 to 0.45)	0.03	1.28 (-1.52 to 4.08)	0.08
1,2,3,7,8,9-HxCDD	-4.55 (-17.2 to 8.11)	0.23	0.05 (-0.60 to 0.70)	0.01	1.28 (-0.78 to 3.33)	0.09
1,2,3,4,6,7,8-HpCDD	7.49 (-3.56 to 18.5)	0.25	-0.03 (-0.60 to 0.55)	0.01	0.62 (-1.21 to 2.46)	0.07
ΣPCDD/F	9.23 (-6.03 to 24.5)	0.24	-0.05 (-0.85 to 0.74)	0.01	0.97 (-1.55 to 3.49)	0.07
PCDD_WHO-TEQ	-13.7 (-30.6 to 3.33)	0.26	-0.15 (-1.04 to 0.74)	0.02	1.37 (-1.45 to 4.20)	0.08
OTCs						
DBT	-6.22 (-34.8 to 22.4)	0.22	-0.37 (-1.84 to 1.10)	0.02	-2.52 (-7.16 to 2.13)	0.08
TPhT	-0.14 (-21.9 to 21.6)	0.22	-0.01 (-1.12 to 1.11)	0.01	-2.24 (-5.75 to 1.27)	0.09
OCPs						
PeCB	4.43 (-9.49 to 18.3)	0.23	0.31 (-0.40 to 1.02)	0.03	0.23 (-2.05 to 2.52)	0.06
α-HCH	1.36 (-4.16 to 6.87)	0.22	0.16 (-0.12 to 0.44)	0.04	0.56 (-0.33 to 1.45)	0.09
(-)-α-HCH	1.18 (-5.01 to 7.37)	0.22	0.18 (-0.14 to 0.49)	0.04	0.57 (-0.44 to 1.57)	0.08

(+)-α-HCH	1.52 (-3.89 to 6.94)	0.22	0.12 (-0.15 to 0.40)	0.03	0.50 (-0.38 to 1.38)	0.08
γ-HCH	10.7 (-4.60 to 25.9)	0.25	-0.33 (-1.12 to 0.46)	0.03	-1.00 (-3.53 to 1.53)	0.07
PCA	3.28 (-5.08 to 11.6)	0.23	-0.12 (-0.55 to 0.31)	0.02	1.07 (-0.27 to 2.42)	0.10
HCB	-6.10 (-29.8 to 17.6)	0.22	-0.74 (-1.94 to 0.46)	0.04	-2.46 (-6.29 to 1.37)	0.09
OC	3.26 (-8.11 to 14.6)	0.22	-0.16 (-0.74 to 0.42)	0.02	-0.29 (-2.16 to 1.57)	0.06
OXC	4.10 (-6.57 to 14.8)	0.23	0.11 (-0.44 to 0.66)	0.02	0.19 (-1.56 to 1.95)	0.06
(+)-OXC	1.38 (-6.77 to 9.53)	0.22	-0.06 (-0.48 to 0.36)	0.02	0.02 (-1.31 to 1.35)	0.06
(-)-OXC	0.48 (-7.04 to 7.99)	0.22	-0.07 (-0.46 to 0.31)	0.02	0.32 (-0.91 to 1.55)	0.07
c-HE	6.50 (-7.19 to 20.2)	0.23	-0.11 (-0.82 to 0.60)	0.02	0.10 (-2.16 to 2.36)	0.06
(+)-HE	4.05 (-5.62 to 13.7)	0.23	0.00 (-0.50 to 0.50)	0.01	0.69 (-0.90 to 2.27)	0.07
(-)-HE	-0.65 (-8.61 to 7.30)	0.22	-0.18 (-0.58 to 0.23)	0.03	-0.27 (-1.57 to 1.04)	0.06
o,p'-DDE	4.09 (-5.94 to 14.1)	0.23	0.03 (-0.49 to 0.54)	0.01	-0.08 (-1.73 to 1.58)	0.06
p,p'-DDE	-7.41 (-17.9 to 3.04)	0.25	-0.31 (-0.85 to 0.23)	0.04	-0.64 (-2.37 to 1.10)	0.07
END-I	2.45 (-7.53 to 12.4)	0.22	0.17 (-0.34 to 0.68)	0.02	-0.54 (-2.17 to 1.10)	0.07
p,p'-DDD	-3.10 (-14.0 to 7.85)	0.22	-0.08 (-0.65 to 0.48)	0.02	-0.98 (-2.76 to 0.80)	0.08
o,p'-DDD	0.59 (-7.07 to 8.25)	0.22	-0.07 (-0.46 to 0.32)	0.02	0.25 (-1.00 to 1.50)	0.06
Dieldrin	7.83 (-4.71 to 20.4)	0.24	0.05 (-0.61 to 0.70)	0.01	-1.51 (-3.55 to 0.54)	0.10
o,p'-DDT	3.20 (-6.94 to 13.3)	0.23	0.05 (-0.47 to 0.57)	0.01	-0.56 (-2.23 to 1.10)	0.07

p,p'-DDT	-0.78 (-11.5 to 9.94)	0.22	-0.01 (-0.56 to 0.54)	0.01	-1.15 (-2.88 to 0.58)	0.09
Mirex	-0.44 (-7.69 to 6.81)	0.22	0.03 (-0.35 to 0.40)	0.01	-0.16 (-1.35 to 1.03)	0.06
ΣOCP	-9.67 (-23.4 to 4.05)	0.25	-0.41 (-1.12 to 0.29)	0.04	-1.15 (-3.41 to 1.12)	0.08

Abbreviations: MBT, monobutyltin; DBT, dibutyltin; TPhT, triphenyltin; PCA, pentachloroanisole; HCB, hexachlorobenzene; OXC, oxychlordane; c-HE, *cis*-heptachloroepoxide; END-I, endosulfan-I.

[a]Placental concentrations of PCDDs, PCDD_WHO-TEQ, non-ortho-PCBs (PCB-81, 77, 126 and 169), and PCB_WHO-TEQ were expressed as pg/g lipid. The concentrations of PBDEs, ortho-PCBs, and OCPs were expressed as ng/g lipid. The concentrations of OTCs were expressed as ng/g fresh weight.

[b]Adjusted for maternal smoking, gestational age, and neonatal birth weight.

[*]$p < 0.05$. [**]$p < 0.01$. [#]$p < 0.10$.

rT_3 was inversely significantly associated with TBT (β=-2.65; 95% CI: -3.79, -1.51; $p < 0.001$) and the sum of OTCs (β=-4.96; 95% CI: -7.78, -2.14; $p < 0.001$), while none of the OTCs showed a significant association with T_4 or T_3.

β-hexachlorocyclohexane (β-HCH) showed negative tendencies with T_4 (β=-13.9; 95% CI: -28.7, 0.90; $p < 0.065$), T_3 (β=-0.68; 95% CI: -1.44, 0.09; $p < 0.081$) and rT_3 (β=-2.06; 95% CI: -4.50, 0.38; $p < 0.097$), although none of them were statistically significant. Methoxychlor (MOC) was inversely significantly associated with rT_3 (β=-2.30; 95% CI: -3.88, -0.72; $p < 0.005$).

In the PCA, the first 3 principal components (PCs) explained 27.8%, 9.3%, and 6% of the variances, respectively (Fig. 3.2).
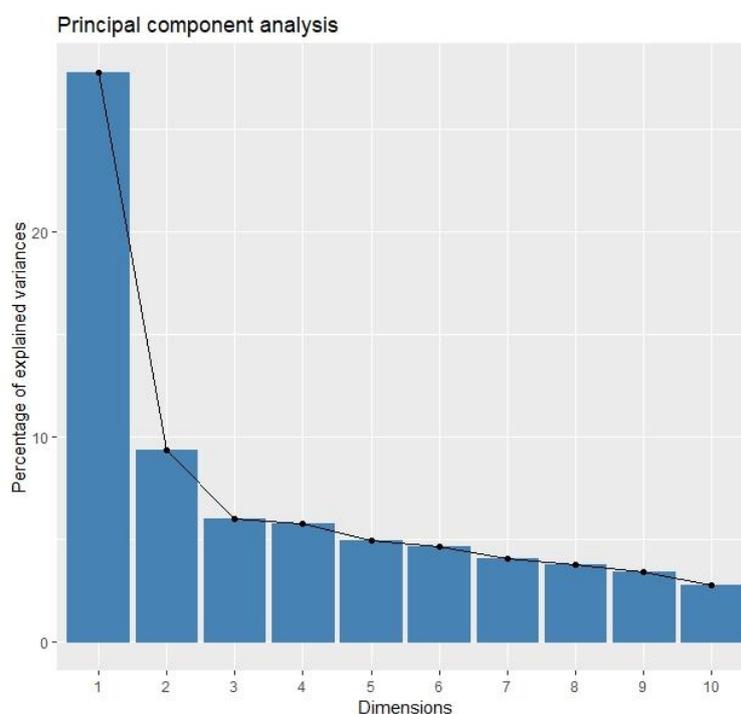
Fig. 3.2 Percentage of explained variances by the first 10 principal components (PCs)

As shown in Fig 3.3, most of the variances in the first PC were contributed by PCBs, while most of the variances in the second PC were contributed by PBDEs. To avoid multicollinearity including all the individual variables in multiple regression models, individual PC scores were included as a "common pregnancy-related vector" in the final model.

Grouped POPs were included in multiple linear regression models based on hierarchical cluster analysis (shown in Fig. 3.4).
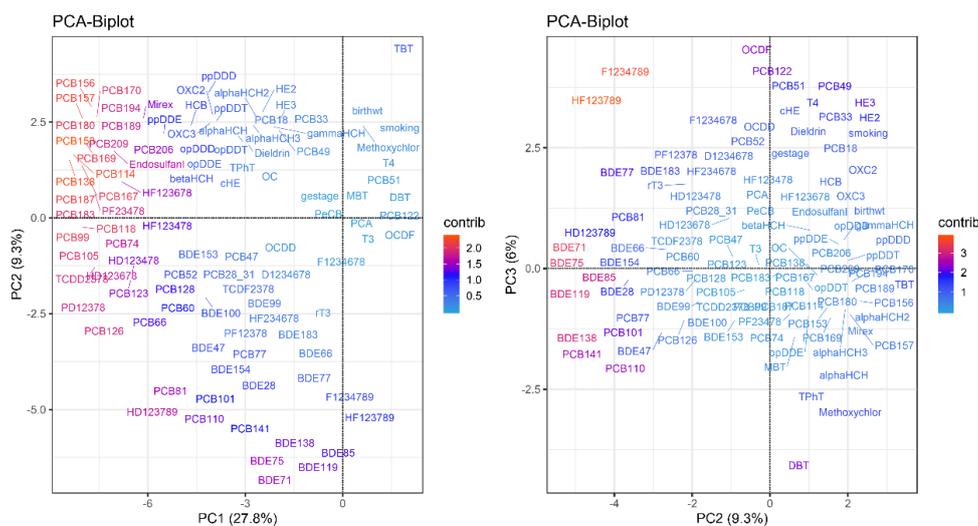
Fig. 3.3 PCA loading plot for THs, POPs, and confounders. The plot describes the contributions of variables on PC1 and PC2.
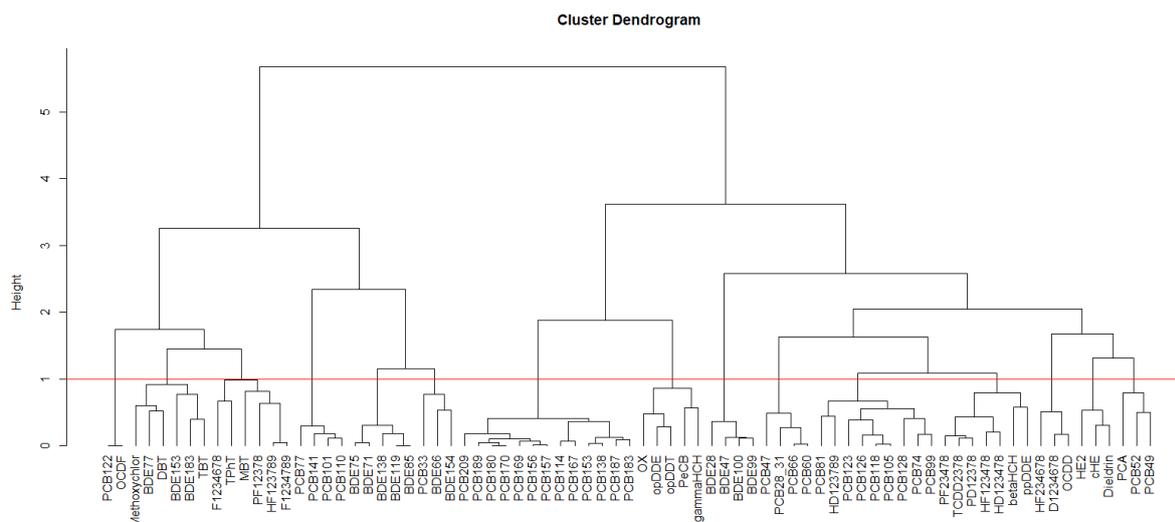
Fig. 3.4 Hierarchical clustering of POPs based on concentrations in 58 placental samples. The figure depicts the hierarchical structure obtained from the correlation between compounds (method: complete linkage).

As shown in Table 3.7, some associations were observed: (a) T_4 was significantly negatively associated with PBDE group (including BDE-28, 47, 99, and 100); (b) T_4 was significantly positively associated with PCDD group (including 2,3,4,6,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDD, and OCDD); (c) Although not significant, T_4 was marginally positively

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associated with OCP group 1 (including OX, o,p'-DDE, o,p'-DDT, PeCB, and γ-HCH), and OCP group 2 (including (-)-HE and Dieldrin);(d) rT_3 was marginally negatively associated with PCDD group (including 2,3,4,7,8-PE, 2,3,7,8-TCDD, 1,2,3,7,8-HpCDD, 1,2,3,4,7,8-HxCDF, 1,2,3,4,7,8-HxCDD, β-HCH, and p,p'-DDE)

Table 3.7 Associations between placental THs and POPs grouped based on hierarchical clustering.

	T_4 (ng/g)		T_3 (ng/g)		rT_3 (ng/g)	
	β (95% CI)	R^2	β (95% CI)	R^2	β (95% CI)	R^2
Group 1	1.59 (-5.91–9.09)	0.24	0.05 (-0.34–0.44)	0.02	0.83 (-0.39–2.04)	0.11
Group 2	-7.08 (-21.1–6.95)	0.25	-0.22 (-0.95–0.51)	0.02	0.05 (-2.28–2.39)	0.07
Group 3	-16.7 (-59.6–26.1)	0.25	-0.91 (-3.13–1.31)	0.03	1.65 (-5.44–8.73)	0.08
Group 4	18.0 (-0.45–36.4)#	0.29	0.12 (-0.86–1.11)	0.01	-1.44 (-4.56–1.67)	0.09
Group 5	-15.3 (-31.3–-0.68)*	0.29	0.03 (-0.83–0.89)	0.01	1.27 (-1.43–3.98)	0.09
Group 6	0.90 (-16.6–18.4)	0.24	0.24 (-0.66–1.14)	0.02	-0.98 (-3.84–1.89)	0.08
Group 7	-12.8 (-33.0–7.33)	0.26	-0.84 (-1.88–0.19)	0.06	-2.74 (-6.03–0.54)#	0.12
Group 8	13.7 (0.70–26.8)*	0.30	-0.02 (-0.72–0.69)	0.01	0.39 (-1.84–2.63)	0.08
Group 9	13.0 (-2.19–28.1)#	0.28	-0.01 (-0.82–0.79)	0.01	-1.78 (-4.30–0.73)	0.11

Group 1: PCB-77, -101, -110, and -141. Group 2: BDE-71, -75, -85, -119, -138. Group 3: PCB-114, -138, -153, -156, -157, -167, -169, -170, -180, -183, -187, -189, and -209. Group 4: OX, o,p'-DDE, o,p'-DDT, PeCB, γ-HCH. Group 5: BDE-28, -47, -99 and -100. Group 6: PCB-28, -31, -47, -60, and -66. Group 7: 2,3,4,7,8-HpCDF, 2,3,7,8-TCDD, 1,2,3,7,8-HpCDD, 1,2,3,4,7,8-HxCDF, 1,2,3,4,7,8-HxCDD, β-HCH, and p,p'-DDE. Group 8: 2,3,4,6,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDD, and OCDD. Group 9: (-)-HE, and dieldrin.

3.4 Discussion

The primary goal of this study was to determine placental concentrations of THs and to examine their associations with various POPs. Background exposures of POPs could interfere with the thyroid homeostasis in placenta, and thereby influence the THs delivered to the fetus, which are critical for both the fetal and neonatal development. Although the effect of each chemical seems scarce, the added effects may cause inappropriate consequences.

We measured T_4, T_3 and rT_3 by an UPLC-Q-TOF-MS method, which offers better sensitivity and specificity than radio immunoassay (RIA)-based methods, as explained previously [181]. The TH concentrations measured in our study corresponded well with previous findings [42, 43].

In utero exposure to POPs occurs during gestation as a result of placental transfer. Thus, placental concentrations of some contaminants may be representative of fetal exposures [182]. The exact mechanisms involved in the transplacental transfer of POPs are not fully understood. Binding of POPs to transthyretin (TTR) and TH membrane transporters may affect this process. Our results revealed a widespread background contamination of POPs in human placenta. BDE-47 and BDE-153 are the major components compared with other BDE congeners, which is in accordance with a previous study [183]. ΣPBDE concentration was lower than those measured in placental samples from South China (mean: 13.3 ng/g lipid; range: 4.32–42.0 ng/g lipid) [90] and the USA (mean: 14.6 ng/g lipid; range: 0.62–521.8 ng/g lipid) [42] due to the lower human exposure of PBDEs in Europe [90]. Wang *et* al. reported lower PCB-concentrations than our results, and PCB-118, 156 and PCB-105 had the highest levels [184]. This difference may be due to the region-specific pollution, as well as the sampling time because the human exposure of PCBs continues to decrease since 1970s. The ΣPCDD/F concentrations were in the same range as in a previous study in placenta from cesarean section [185]. The placental levels of OTCs were in the same range with our previous study [178].

We observed positive correlations between T_4 and gestational age, maternal smoking, and birth weight, which were in accordance with previous studies [186-189]. Therefore, these characteristics were included in the statistical analysis as cofounders. However, some other researchers have also found associations of THs with mode of delivery [186], alcohol consumption [190], and maternal BMI [191].

We investigated the possible relationship between THs and cryptorchidism because a previous review suggested that THs may play a role in the development and function of the testis [192]. Multiple logistic regression revealed no difference of THs between the two groups, which could be due to the small number of cases. Bruker-Davis *et* al. also reported no difference of THs in cord blood from mothers that gave birth to boys with and without cryptorchidism

[193]. However, the results for analyzing the effect of THs may be very different when a different matrix (i.e., maternal serum, cord blood, infant serum or placenta) and different TH measurements (i.e., total concentrations or free concentrations) were used.

This study revealed that POP exposures in general result in lower levels of total T_4 or increased levels of total T_3 and total rT_3. Previous studies have reported positive [81, 82, 194], negative [82, 194], and no associations [61] between certain POPs and TH concentrations in pregnant women and newborns. However, our results may not be directly comparable to these studies because the biological matrix for exposure measurements and TH determination is not the same. Only one study exists on THs and brominated flame retardants in placenta, in which a sex-specific manner of association was observed: placental BDE-99 and BDE-209 were negatively associated with rT_3 levels in male infants, while BDE-99 and 2,4,6-tribromophenol (2,4,6-TBP) were positively associated with T_3 concentrations in female infants [42].

The mechanisms involved in the thyroid-disrupting process of POPs are diverse and complex: (1) POPs may disrupt the activity of the thyroid gland by interference with the thyroid-stimulating hormone (TSH)-receptor, sodium iodide symporter (NIS), thyroid peroxidase (TPO), as well as other receptors on the thyrocyte; (2) POPs may competitively bind to TH binding proteins, i.e., thyroid binding globulin (TBG), transthyretin (TTR) and thyroid hormone receptors (TR); (3) POPs may affect the peripheral TH metabolism and clearance by activation or inactivation of the enzymes, i.e., iodothyronine deiodinases and UDP-glucuronosyltransferases (UDPGTs) [70]. In blood, certain POPs and their hydroxylated metabolites may competitively bind with TH-binding proteins and deiodinases, leading to an increase in the free T_4 concentration. The feedback regulation via TSH may compensate for this change, resulting in a stable concentration of T_4 in serum [70]. Total T_4 in placenta mainly originates from the transplacental passage of maternal free T_4, while total T_3 and total rT_3 were derived entirely from placental and foetal metabolism of T_4 (deiodinases D2 and D3) [88]. Disrupting the TH-protein (i.e., TTR and TH membrane transporters) binding and metabolism in placenta may be of significance. TTR plays a crucial role in transferring free T_4 across the placenta to the fetal compartment [70]. Binding of certain POPs to TTR may facilitate the transport of these compounds, while reducing T_4 delivery to the fetus. Different compounds

exhibit different affinities to these proteins. PBDEs, PCBs, and especially their hydroxylated metabolites have a high degree of structural resemblance of T_4 and are therefore able to competitively bind with TTR.

3.4.1 Associations of concentrations of T_4 with POPs

Our results revealed inverse associations of BDE-47, -99, -100 and ΣPBDE with T_4, which were in accordance with a previous report [42]. This could be due to the interference of these chemicals with the TH transport system because strong affinities between TTR and BDE-47, 99, and BDE-100 have been observed previously [195]. PCBs were expected to show associations with T_4 levels because they have a high degree of structural resemblance to T_4. Boas *et* al. also suggested that perinatal exposure to PCBs decrease THs [70]. However, only negative tendencies between T_4 and PCB-99, 118 and PCB-167 were found in this study. This might be due to the differences in the applied matrix, as well as the differences in biological indicators of exposure between different studies. For example, Majidi *et* al. analyzed the TH effect of PCBs using standardized concentrations expressed in total PCB equivalent per kg of lipids in maternal plasma (μg PCB$_{MPEQ}$/kg lipid). The results suggested little evidence for the impact of PCBs on thyroid function in pregnant women and newborns [196]. In addition to TTR, PCDD/Fs are able to bind to and activate the aryl hydrocarbon receptor (AhR), inducing UDPGT as well as cytochrome P450 enzymes, which stimulate the glucuronidation of T_4 and biliary excretion of this conjugate, resulting in lower levels of T_4 [197]. This may explain the inverse association of 2,3,7,8-TCDD with T_4 observed here. However, we also observed positive associations between T_4 and 1,2,3,7,8-PeCDD, 1,2,3,4,6,7,8-HpCDF, and OCDD here, probably due to the inhibitory effect of dioxins on the activity of placental deiodinases. Human and animal studies revealed negative associations between T_4 levels and pesticides such as dichlorodiphenyltrichloroethane (DDT) (and its metabolite DDE) and hexachlorobenzene (HCB). Interference with the thyroid peroxidase (TPO) activity and binding protein are possible mechanisms [70]. β-HCH was negatively associated with T_4 in this study. This might be explained by the similar bioconcentration factor of β-HCH and HCB, which may lead to a similar mechanism of action.

3.4.2 Associations of concentrations of rT_3 with POPs

rT_3 is an inactive metabolite of T_4 formed by deiodination. Inhibition of Dio activities by POPs may lead to reduction in T_4 metabolism, causing lower levels of placental rT_3 [198]. For example, Leonetti *et* al. reported a negative association between rT_3 and BDE-99 among males [42]. However, we did not see a significant association between BDE-99 and rT_3 here, probably because of the differences in study design and sampling methodology. Our study found that TBT, ΣOTC, β-HCH, and MOC were inversely associated with rT_3. Besides, inhibition of the DIO activities can lead to an increase of T_4 levels, which was not observed in our study. We also found positive associations between rT_3 and PCB-81, -101, -183, 1,2,3,7,8-PeCDF, 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 2,3,4,6,7,8-HxCDF, and 1,2,3,4,6,7,8-HpCDF, which suggested an alternative mechanism of action. Interestingly, the association between rT_3 and OTC placental exposure was assessed for the first time. OTCs are a group of chemicals used as biocides in antifouling paints. TBT can cross the placenta, inducing physiological and morphological changes, resulting in abnormal fetal and postnatal development [199]. The present study suggested that *in utero* exposure to OTC, especially TBT, leads to lower level of rT_3.

3.4.3 Associations of concentrations of T_3 with POPs

We found only a few associations between POPs and the biologically active thyroid hormone, T_3. Among the 82 POPs investigated, T_3 only showed positive associations with 2,3,7,8-TCDF and 1,2,3,7,8-PeCDF, and negative tendency with β-HCH, suggesting a low sensitivity of T_3 homeostasis for POP exposures. Placental T_3 is produced by deiodination in the placenta and foetus, whereas T_4 is partly derived from the transplacental passage of maternal free T_4. Therefore, the feedback regulation, which is induced by T_3, may not take place in placenta, and thus maternal pituitary regulation might be out of regulatory function for placental T_3.

We also estimated the associations of THs with the WHO-TEQ values of PCDD/Fs (PCDD/F_WHO-TEQ) and PCBs (PCB_WHO-TEQ). As shown in Table 14, no significant associations were observed.

3.4.4 Strengths and limitations

This study has several unique strengths: (a) This is the first study investigating thyroid-disrupting effect of as many as 82 POPs, as well as the WHO-TEQ values of PCDD/Fs and PCBs, which therefore provides an overview of the possible relationships between the ubiquitous POPs and THs in placenta; (b) UPLC-Q-TOF-MS was adopted for TH analysis. This approach provides better accuracy and reliability than RIA methods used in previous studies, producing more reliable results; (c) A wide variety of demographical characteristics were assessed and considered in the statistical analysis. Some of these variables could influence the THs during pregnancy. Taking various characteristics into consideration increases the robustness of the analysis. However, the study also has certain limitations. For example, the number of samples is limited (n=58), which may reduce the statistical power. Only placenta samples from mothers that gave birth to boys were included in this research, thus we could not reveal any sex-dependent effect. The hydroxylated metabolites of certain POPs (e.g., OH-PCBs and OH-PBDEs), which generally show higher affinities in binding with the TH-binding proteins than the mother compound, were not included in this study. The TH and POP concentrations in this study reflect the situation at delivery instead of the exposure during the entire pregnancy. Our previous study observed considerably higher concentrations of PBDEs per gram fat in breast milk than in placenta and suggested that milk analyses might be more reliable toward the lower end of concentrations [174]. Additionally, free THs may be more important for analyzing the effects of POP exposure, while total TH concentrations were measured in this study.

Finally, with as many as 82 POP congeners, their sums and 3 THs, some significant associations may occur by chance. However, in particular the associations between PBDEs and T_4 appear robust, as all associations showed the same direction and there is a well-known structure resemblance of PBDEs to T_4.

3.5 Conclusion

In summary, our results suggest that background exposure to POPs can alter thyroid homeostasis in pregnant women, subsequently affecting the thyroid homeostasis in placenta.

Our results highlight the challenges of assessing effects on thyroid function, especially during pregnancy, due to the complexity of contaminant mixtures and the sensitivity of the thyroid system of the pregnant woman and the fetus. Finally, the results of this study should be interpreted with caution due to the limited number of subjects included in the analysis. The findings should be confirmed with more placenta samples from both boys and girls, also including the DIO enzyme activities and the hydroxylated metabolites of PBDEs and PCBs.

Chapter 4 Persistent organic pollutants in human breast milk and associations with maternal thyroid hormone homeostasis

Abstract

Recent studies have suggested the thyroid-disrupting effects of POPs. However, the associations of low-exposure POPs with THs remain unclear. In this chapter, we aim to assess the associations of low exposure of POPs, including PBDEs, PCBs, PCDD/Fs, and PBDD/Fs), with THs (total T_4 (TT_4), total T_3 (TT_3), and total rT_3 (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.

collection, data interpretation, and manuscript review.

4.1 Introduction

In this chapter we evaluated the associations between POPs (PCBs, PBDEs, PCDD/Fs, and PBDD/Fs) and THs (TT_4, TT_3, and TrT_3) in human breast milk collected from the LUPE (Länderuntersuchungsprogramme) study (2015-2016, Bavaria, Germany). We firstly optimized a method for TH determination using HPLC-MS/MS). Single-pollutant regression and multipollutant models such as principal component analysis (PCA), partial least squares (PLS), and hierarchical clustering were employed to evaluate the associations of THs with POPs [200].

4.2 Materials and methods

4.2.1 Sample collection

We collected 99 human breast milk samples from the Länderuntersuchungsprogramme (LUPE) study, which is a prospective German cohort study established to assess human exposure to POPs and health outcomes using human breast milk. 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 immediately 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 °C until processing.

This study was approved by the ethics committee of the Bavarian Chamber of Physician. Written informed consent was obtained from all participants.

4.2.2 POP analysis

Detailed analytical methods regarding POP quantificaiton are available elsewhere [201, 202]. 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. For each POP category, we calculated total PBDEs, total PCBs, total PCDD/Fs, and total PBDD/Fs as the sum of detectable levels of all chemicals within that class. Additionally, we also calculated the WHO$_{2005}$-TEQ, which was developed to assess the toxicity of dioxins and dioxin-like PCBs (dl-PCBs) relative to 2,3,7,8-TCDD [203].

4.2.3 TH measurement

(1) Chemicals and reagents

The chemicals, reagents, and materials used in this study have been illustrated in Chapter 2.

(2) Preparation of standard solutions

A stock solution of each TH standard (100 ng/mL for T_4, T_3, rT_3, 3,3'-T_2, 3-T_1AM, $^{13}C_6$-T_4, $^{13}C_6$-T_3 and $^{13}C_6$-rT_3, 50 ng/mL for 3,5-T_2, T_1, T_0 and $^{13}C_{12}$-T_4) was prepared using 0.1 N NH$_4$OH in MeOH and stored at -20 ℃. Calibration standard solutions (0–150 ng/mL of each analyte and 10 ng/mL of each quantification standard) were prepared from individual stock solutions through dilution using 0.1% formic acid in ACN: H$_2$O (2:8, v/v).

(3) Instrumentation

Quantification of THs was performed using an Agilent 6470 triple quadrupole tandem mass spectrometry (LC-MS/MS) system connected with Agilent 1290 Infinity II LC system. Optimized MS/MS parameters are shown in Table 4.1. The injection volume for LC-MS/MS analysis was 20 μL. Data acquisition and data analysis were performed using Agilent MassHunter Workstation software. The mobile phases of gradient HPLC method were water (A) and ACN (B) each containing 0.1% formic acid (v/v). The following gradient program was adopted: 5% B kept for 3 min, ramped linearly to 30% B in 1 min, then gradually increased to 38% B in 2.5 min and kept for 1 min, followed by an increase to 40% B in 1.5 min and held for 1 min, then increased to 100% B in 2 min and kept for 1 min to remove lipophilic components, finally returned to initial conditions in 0.2 min. Further 3.8 min was allowed for re-equilibration before the next injection.

Table 4.1 Optimized MS/MS parameters for thyroid hormones. For each compound SRM ion-transitions are shown as m/z for parent ion and two product ions (for quantification and confirmation). Compound optimized values for retention time (t_R), fragmentor (F), collision energy (CE), collision acceleration voltage (CAV) and dwell times. Quantification standards.

Compound	t_R (min)	Parent ion (m/z)	Product ions (m/z)	F (V)	CE (V)	CAV (V)	Dwell (msec)
Target compounds							
T_4	7.49	777.4	731.4	140	25	1	50
			633.4	140	25	1	50
T_3	6.70	651.8	605.6	120	25	1	50
			507.6	120	25	1	50
rT_3	6.96	651.8	605.6	120	25	1	50
			507.6	120	25	1	50
$3,3'$-T_2	6.27	525.8	479.8	90	15	2	50
			381.8	90	15	2	50
$3,5$-T_2	5.91	525.8	479.8	90	15	2	50
			381.8	90	15	2	50
T_1	5.68	399.9	353.8	90	12	1	50
			256	90	15	1	50
3-T_1AM	5.79	356	339	80	18	1	50
Quantification standards							
ML-T_4	7.49	783.6	737.5	140	25	1	50
ML-T_3	6.70	657.7	611.6	120	25	1	50
ML-rT_3	6.96	657.7	611.6	120	25	1	50
ML-$3,3'$-T_2	6.27	531.8	485.8	100	25	2	50
ML-T_1AM	5.79	362	345	104	12	1	50

Abbreviations: t_R, retention time. F, fragmentor. CE, collision energy. CAV, cell accelerator voltage.

(4) Sample preparation

The optimized sample preparation procedure is shown in Fig. 4.1. Briefly, the process contains three clean-up processes: protein-precipitation, lipid-elimination, and SPE extraction. For protein elimination, MeOH containing 3% formic acid was incubated with breast milk, while $CHCl_3$: MeOH (2:1, v/v) was used for extracting THs from infant formula. Lipids was removed by extracting with 0.05% $CaCl_2$ solution, which has been described before [37, 168]. Finally, the solution was further extracted with a weak cation-exchangers SPE cartridge (Bond Elut Plexa PCX).

Breast milk   **Infant formula**

```
        Breast milk                              Infant formula

   ┌──────────────────┐                    ┌──────────────────────┐
   │ 200 µL breast milk │                   │ 100 mg infant formula │
   └──────────────────┘                    └──────────────────────┘
       │ Spike of quantification standards      │ Spike of quantification standards
   ┌───────────────────────────┐            ┌─────────────────────────────────┐
   │ Addition of 1 mL MeOH (3% HCOOH) │      │ Addition of 500 µL MeOH and 1 mL CHCl3 │
   └───────────────────────────┘            └─────────────────────────────────┘
       │ Vortex. Equilibration: 1 h on ice      │ Vortex. Equilibration: 1 h on ice
       │ Centrifugation: 9000 rpm, 4 °C, 10 min │ Centrifugation: 5000 rpm, 4 °C, 10 min
   ┌────────────────────────────────┐       ┌────────────────────────────────┐
   │ Supernatant transferred into a new tube │ │ Supernatant transferred into a new tube │
   └────────────────────────────────┘       └────────────────────────────────┘
       │ Repeat twice with 500 µL MeOH          │ Repeat twice with 500 µL MeOH and
       │ (3% HCOOH)                             │ 1 mL CHCl3
   ┌──────────────────────────┐
   │ Addition of 4 mL CHCl3   │
   └──────────────────────────┘
```

Fig. 4.1 Thyroid hormone extraction procedures in human breast milk and infant formula.

Flow continues:
- Addition of 1 mL 0.5% $CaCl_2$
  - Vortex. Centrifugation: 5000 rpm, 4°C, 10min
- Supernatant transferred into a new tube
  - Repeat twice with 1 mL MeOH and 1 mL 0.5% $CaCl_2$
- Addition of 300 µL 10 mg/mL protection solution
  - Vortex
- Addition of 100 µL H_3PO_4
  - Vortex. Centrifugation: 5000 rpm, 4 °C, 5 min
- Clean up with Bond Elut Plexa PCX cartridge
  - Wash: 3 mL 2% HCOOH and 3 mL MeOH: ACN = 1:1 (v/v)
  - Elution: 1 mL 5% NH_3 in MeOH: ACN =1:1 (v/v)
- Evaporate to dryness and redissolve
  - Spike of recovery standard
- LC-MS

(5) Method evaluation

The method was evaluated by intra-day and inter-day variation, spike-recoveries, method limits of detection and quantifications, and matrix effects. The methods for calculating these parameters have been reported elsewhere [37]. The validation parameters are shown in Table 4.2.

(6) Quality assurance and quality control

Instrumental quality control included regular injection of solvent blanks and standard solutions. The analytes identification was based on the retention times compared with quantification standards (not allowed to deviate more than 1.0%) and *m/z* ratios of the selected ions. The maximum allowed analyte mass error employed in this study was 0.3 Da. Moreover, we monitored the recovery of $^{13}C_6$-T_4 quantification standard by spiking 0.6 ng of $^{13}C_{12}$-T_4 to each sample before injection. Compared with analytical standards in neat solution, samples that have high deviation (> 40%) on the peak area of quantification standards or lower recovery of recovery standard (< 40%) were discarded and reanalyzed.

4.2.4 POP analysis

Detailed analytical methods regarding POP quantificaiton are available elsewhere [201, 202]. 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 [203]. 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.

4.2.5 Statistical analysis

The statistical analyses were performed on POP congeners showing a detection frequency (DF) above 80%, measurements below the LOQ were replaced by LOQ×DF [204]. The POPs with DF 30–80% were used as dichotomized variable (detected vs not detected) for statistical analyses. Normality was tested using Shapiro-Wilk test. The distributions of biomarkers were log-normal and therefore transformed by the natural logarithm. Then, the POPs were normalized by expressing each exposure as its z-score, which was calculated by subtracting the mean and dividing by the standard deviation for each biomarker. 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. All statistical analyses were conducted using R (version 3.4.2; R Foundation for Statistical Computing, Vienna, Austria). 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. 4.2)
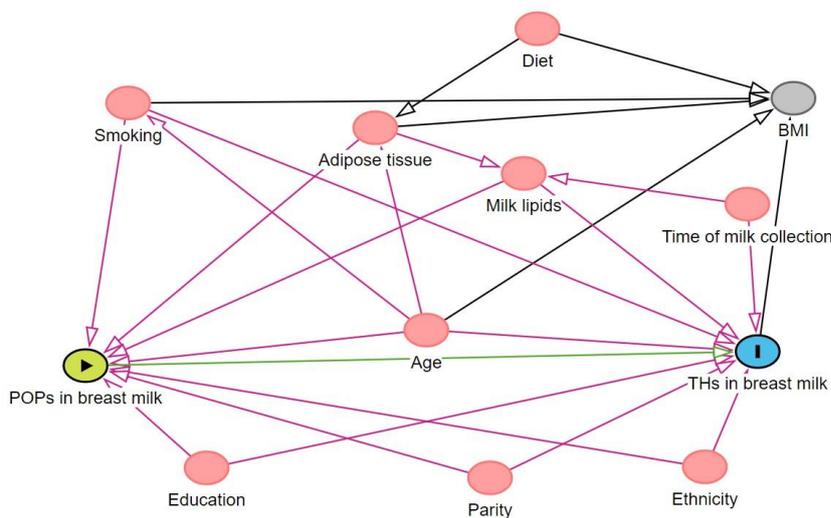
Fig. 4.2 Directed acyclic graph (DAG) illustrating relationships between milk POPs, THs, and covariates.

(1) Covariates

Potential confounders considered for inclusion in models include maternal age (years), body mass index (BMI), educational level (≥ 12 vs < 12 years), parity, smoking (yes vs no), diet (vegetarian vs normal diet), infant gender, infant age at sampling (days). Confounders were identified based on previous reports and a directed acyclic graph (DAG) framework (Fig. 4.2). Data on most covariates were complete. Values of missing covariates were imputed at random based on observed probability distributions (< 2% missing) or on prediction models using non-missing variables (≥ 2% missing). The scenarios include variables known to influence THs (e.g., smoking [73], parity) or breast milk lipid levels (e.g., body mass index, parity, infant age at sampling).

(2) Single pollutant model

Linearity of the associations between POPs and THs were examined using generalized additive models (GAM). Some of the POP congeners showed significant non-linear associations with THs, thus we modeled all exposure biomarkers in categories defined by tertiles.

(3) Principal component analysis

Interpretation of the effect of individual POP congeners can be misleading because of the structural and biological similarity within and across the classes. The multiple collinearity was assessed by the eigen values of the correlation and the variable inflation factor (VIF). PCA is a multivariate approach that is useful to convert the correlated variables into a set of principal components (PCs). The first PC can explain the most variation of the original data, the second PC explains the most amount of the remaining variation. The resulting principal components with eigenvalue of > 1 were linked to THs by the same models as described for the single pollutant models, and adjusted for confounders and statistically significant covariates. The number of factors was decided based on the scree plot [205]. Varimax rotation was applied to calculate factor scores for each participant. The factor scores were categorized into tertiles and included in the regression models. Regressions were performed including factors separately and simultaneously.

(4) Hierarchical clustering

We used PLS regressions to estimate the impact of all POPs and covariates simultaneously on THs. To reduce data and increase the model predictive ability, only variables with variable importance to projection (VIP) values > 0.4 were included in the final model. The score of each woman on the PC 1 was included as a common vector in multiple linear regression models to avoid collinearity issues while adjusting for these factors. In order to minimize the number of contaminants 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.

(5) Sensitivity analyses

Previous studies measuring exposure biomarkers in serum typically adjust for lipid content. However, there is controversy regarding the best approach. In this study we performed the analyses including POP in units of nanograms per gram lipid. In sensitivity analysis we repeated the analyses with POPs in units of nanograms per liter milk and included lipid content as a covariate in the regression model [206]. Another sensitivity analysis was conducted using the subset of exposure biomarkers with the highest sample size and as many exposure biomarkers as possible.

4.3 Results

4.3.1 Biomarker concentrations and their correlations

A method based on LC-MS/MS was optimized for TH quantification. As shown in Table 4.2, 4.3, and Fig. 4.1, 4.3 and 4.4, 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 4.2). Representative MRM chromatograms of THs found in human breast milk are shown in Fig. 4.3 & 4.4 Table 4.3 shows the method validation parameters in infant formula. The mean ± SD concentrations were 0.57 ± 0.20, 0.13 ± 0.03, and 0.02 ± 0.01 ng/mL for TT_4, TT_3, and TrT_3, respectively (Table 4.4).

Table 4.2 Method evaluation parameters of the optimized method for analyzing THs in human breast milk.

	T$_4$	T$_3$	rT$_3$	3,3'-T$_2$	3,5-T$_2$	T$_1$	3-T$_1$AM
MDL (ng/mL)	0.06	0.08	0.09	0.09	0.08	0.09	0.13
MQL (ng/mL)	0.20	0.26	0.31	0.30	0.26	0.30	0.42
Matrix effect (%)	-9.67	2.13	4.98	-0.37	-1.11	3.26	14.7
Overall recovery (%)	117	102	103	110	117	125	119
Spike-recovery (%), n=3							
1.5 (ng/mL)	102 ± 5	105 ± 6	111 ± 3	107 ± 1	112 ± 6	104 ± 2	122 ± 3
15 (ng/mL)	108 ± 2	106 ± 3	109 ± 3	102 ± 2	104 ± 7	112 ± 6	115 ± 5
30 (ng/mL)	102 ± 5	104 ± 2	107 ± 1	99.7 ± 4.5	98.4 ± 6.1	107 ± 6	115 ± 5
Intra-day variation (%), n=3							
1.5 (ng/mL)	0.47	4.17	2.53	0.97	5.06	1.74	2.25
15 (ng/mL)	1.12	2.33	2.89	2.07	6.91	5.18	4.57
30 (ng/mL)	3.55	1.60	1.22	4.52	6.24	5.86	4.28
Inter-day variation (%), n=6							
1.5 (ng/mL)	1.56	3.04	4.10	4.89	7.71	6.13	7.18
15 (ng/mL)	4.14	1.37	2.96	1.61	4.39	8.13	5.02

Abbreviations: MDL: method detection limit, MQL: method quantification limit.

Table 4.3 Method evaluation parameters of the optimized method for analyzing THs in infant formula.

	T_4	T_3	rT_3	T_2	rT_2	T_1	T_1AM
MDL (ng/g)	0.07	0.09	0.12	0.08	0.11	0.20	0.17
MQL (ng/g)	0.24	0.31	0.42	0.27	0.37	0.68	0.55
Matrix effect (%)	4.93	0.86	7.02	-0.89	-3.28	-4.43	-4.90
Overall recovery (%)	103	95.5	99.1	98.0	84.0	78.7	102
Spike-recovery (%), n=3							
3 (ng/g)	106 ± 6	85.1 ± 5.1	105 ± 7	98.7 ± 1.6	103 ± 7	73.7 ± 3.4	98.2 ± 1.1
30 (ng/g)	105 ± 1	104 ± 3	108 ± 1	105 ± 2	88.5 ± 3.8	71.3 ± 2.6	96.9 ± 1.2
60 (ng/g)	105 ± 3	96.0 ± 1.2	102 ± 2	97.0 ± 1.4	81.6 ± 0.7	71.1 ± 1.8	88.7 ± 0.7
Intra-day variation (%), n=3							
3 (ng/g)	3.83	3.35	6.31	1.58	6.42	4.67	1.08
30 (ng/g)	0.55	2.81	0.86	1.92	4.25	3.69	1.21
60 (ng/g)	2.38	1.16	1.57	1.40	0.83	2.60	0.82
Inter-day variation (%), n=6							
3 (ng/g)	6.09	6.04	6.13	4.14	6.32	8.30	7.08
30 (ng/g)	6.02	3.60	4.28	4.45	9.80	10.7	8.06

Abbreviations: MDL: method detection limit, MQL: method quantification limit.
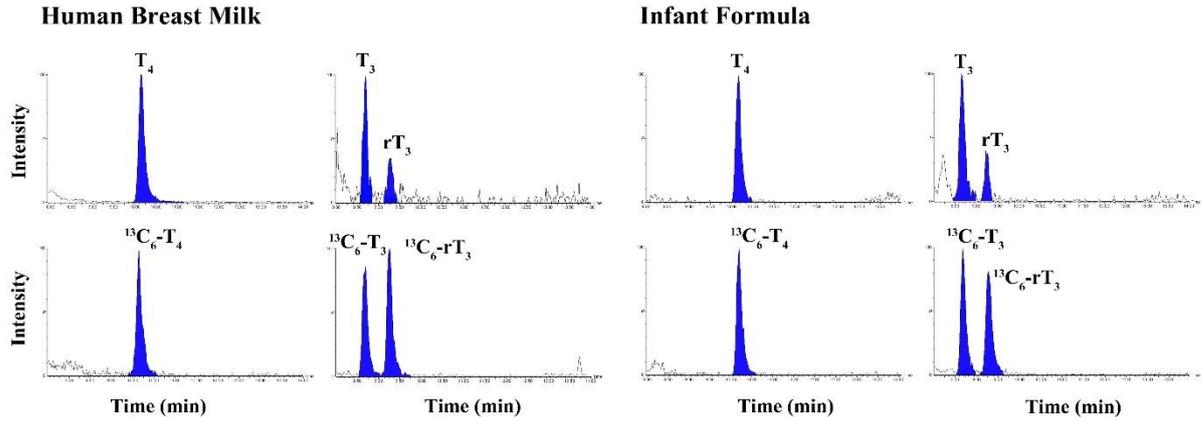
Fig. 4.3 Representative EIC chromatograms of thyroid hormones detected in human breast milk and infant formula using UPLC-Q-TOF-MS.
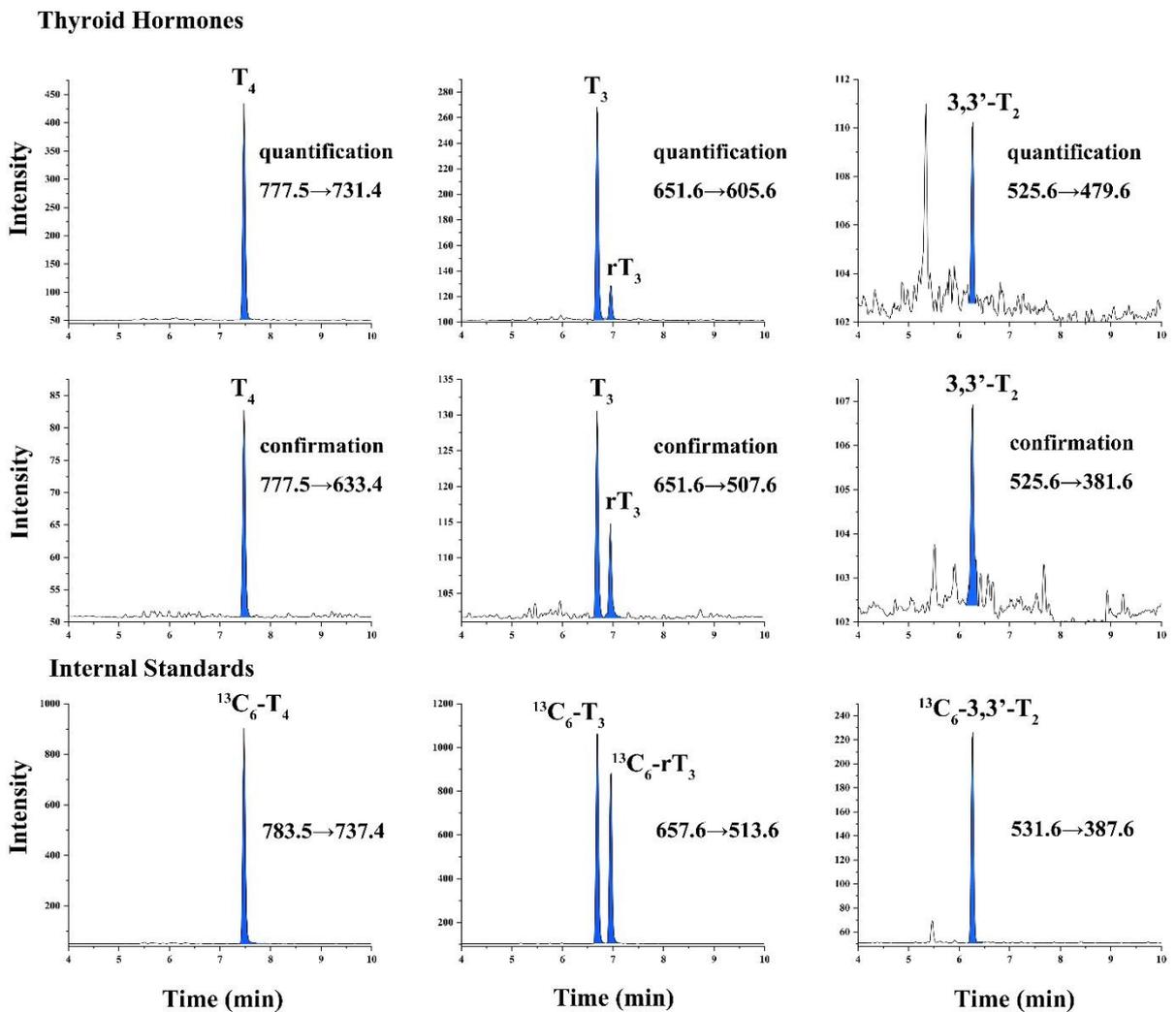
Fig. 4.4 Representative MRM chromatograms of thyroid hormones detected in human breast milk using HPLC-QqQ-MS.

Table 4.4 Concentrations of THs in human breast milk measured in this study and those reported previously (Concentrations of 3,3'-T_2, 3,5-T_2, T_1, and 3-T_1AM were < MDL).

T_4 (µg/L)	T_3 (µg/L)	rT_3 (µg/L)	Method	Sample	Ref.
0.57 ± 0.20	0.13 ± 0.03	0.02 ± 0.01	LC-MS/MS	Breast milk	This study
0.86 ± 0.38	0.14 (0.08–0.18)	n.p.	RIA	Preterm breast-milk	[44]
4.98 ± 1.96	n.p.	n.p.	RIA	Term breast-milk	[44]
29.6 ± 15.5	0.35 ± 0.20	n.p.	CIA	Pooled milk sample from patients with thyroid-related diseases	[207]

Abbreviation. n.p.: not reported. [a]the concentration of T_3 was reported as ng/g of cow milk.

As shown in Table 4.5, 14 PBDEs were detected in more than 80% of milk samples; BDE-209 (median: 440 pg/g lipid weight (lw)) was the dominating compound, followed by BDE-153 (377 pg/g lw), BDE-47 (204 pg/g lw), BDE-197 (73.1 pg/g lw), BDE-99 (62.5 pg/g lw), BDE-207 (56.3 pg/g lw), BDE-100 (54.3 pg/g lw), BDE-28 (29.3 pg/g lw), BDE-206 (28.9 pg/g lw), BDE-183 (28.4 pg/g lw), BDE-208 (19.3 pg/g lw), BDE-196 (16.8 pg/g lw), BDE-203 (16.7 pg/g lw), and BDE-154 (8.25 pg/g lw).

Seventeen PCBs were detected in > 80% of milk samples (Table 4.5). The highest median lipid-based concentration was found for PCB-118 (3619 pg/g lw) followed by PCB-156 (2128 pg/g lw), PCB-167 (662 pg/g lw), PCB-105 (591 pg/g lw), PCB-157 (344 pg/g lw), PCB-114 (224 pg/g lw), PCB-189 (219 pg/g lw), PCB-123 (37.6 pg/g lw), PCB-153 (23.3 pg/g lw), PCB-126 (18.1 pg/g lw), BDE-203 (16.7 pg/g lw), PCB-138 (14.0 pg/g lw), PCB-169 (10.9 pg/g lw), PCB-180 (8.76 pg/g lw), PCB-77 (2.97 pg/g lw), PCB-28 (0.81 pg/g lw), PCB-101 (0.25 pg/g lw), and PCB-52 (0.14 pg/g lw).

Five PCDD/Fs were quantified in > 80% of milk samples. The highest lipid-based concentrations were found for OCDD (17.0 pg/g lw) followed by 2,3,4,7,8-PeCDF (3.80 pg/g lw), 1,2,3,6,7,8-HxCDD (2.94 pg/g lw), 1,2,3,4,6,7,8-HpCDD (2.62 pg/g lw), and 1,2,3,6,7,8-HxCDF (1.03 pg/g lw). The detection frequencies of all the PBDD/F congeners were ≤ 38% and therefore not included in the statistical analyses (shown in Table 4.5).
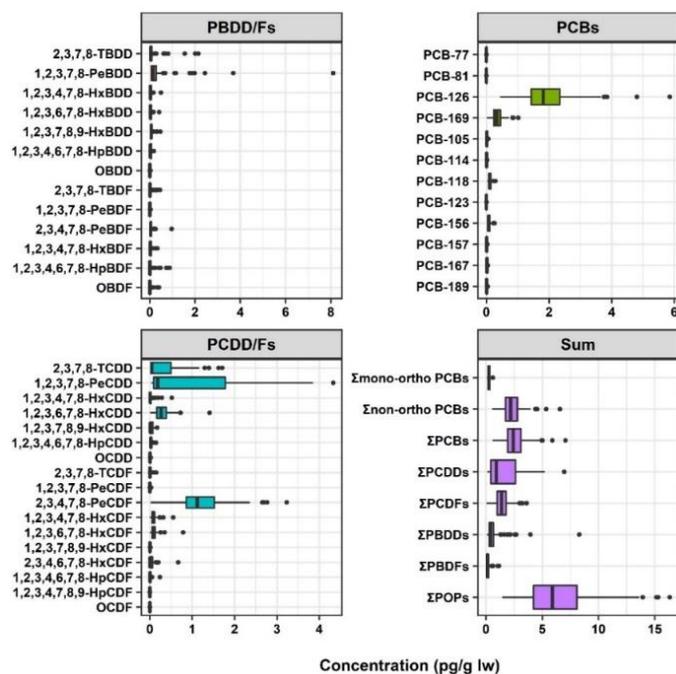
Fig. 4.5 WHO$_{2005}$-TEQ values of PCDD/Fs, PBDD/Fs, and dioxin-like PCBs (pg TEQ/g lw) measured in breast milk from LUMP study.

Fig. 4.5 and Table 4.6 summarize the WHO$_{2005}$-TEQ values of dl-PCBs, PBDD/Fs, and PCDD/Fs. The highest levels in PCBs were found for PCB-126 (1.81 pg/g lw) followed by PCB-169 (0.33 pg/g lw). The highest levels of PCDD/Fs were found for 2,3,4,7,8-PeCDF. 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 4.7-4.9 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.

As shown in Fig. 4.6, THs show 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 were -0.05–0.90, 0.0001–0.98, and 0.18–0.68 for PBDEs, PCBs, and PCDD/Fs, respectively.

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Table 4.5 Distribution of polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), and polybrominated dibenzo-*p*-dioxins and furans (PBDD/Fs) in breast milk from LUPE study (2015-2016, Bavaria, Germany).

POPs	LOQ (pg/mL)	N (%)	Mean pg/mL (pg/g lw)	Min pg/mL (pg/g lw)	Q1 pg/mL (pg/g lw)	Q2 pg/mL (pg/g lw)	Q3 pg/mL (pg/g lw)	Max pg/mL (pg/g lw)
BDE-17	0.05	6 (6)	0.09 (3.85)	< LOQ	< LOQ	< LOQ	< LOQ	0.19 (7.20)
BDE-28	0.05	95 (96)	0.95 (31.7)	< LOQ	0.49 (20.2)	0.81 (29.3)	1.21 (37.7)	4.44 (122)
BDE-47	< 1.46	99 (100)	9.34 (307)	1.46 (61.6)	3.94 (136)	5.88 (204)	9.69 (299)	82.4 (2419)
BDE-66	0.21	39 (39)	0.14 (5.69)	< LOQ	< LOQ	0.09 (4.20)	0.19 (6.20)	0.49 (21.9)
BDE-85	0.09	18 (18)	0.36 (10.9)	< LOQ	0.10 (4.73)	0.20 (9.30)	0.57 (17.6)	1.48 (27.5)
BDE-99	0.46	98 (99)	2.54 (85.8)	< LOQ	1.34 (46.1)	1.82 (62.5)	2.56 (93.9)	17.3 (419)
BDE-100	0.30	97 (98)	2.25 (73.7)	< LOQ	0.93 (31.3)	1.57 (54.3)	2.80 (92.5)	12.0 (364)
BDE-153	< 2.12	99 (100)	14.5 (460)	2.12 (112)	7.50 (304)	11.6 (377)	16.5 (545)	99.0 (1979)
BDE-154	0.28	82 (83)	0.27 (9.42)	< LOQ	0.15 (5.58)	0.23 (8.25)	0.36 (11.1)	0.97 (28.5)
BDE-183	0.03	97 (98)	1.03 (33.7)	< LOQ	0.55 (19.4)	0.86 (28.4)	1.13 (42.2)	6.28 (182)
BDE-196	0.09	89 (90)	0.64 (22.4)	< LOQ	0.35 (12.4)	0.47 (16.8)	0.73 (24.1)	4.55 (146)
BDE-197	< 0.31	99 (100)	2.50 (83.2)	0.31 (19.3)	1.58 (53.6)	2.18 (73.1)	3.19 (103)	7.92 (224)
BDE-203	0.10	91 (92)	0.64 (22.8)	< LOQ	0.33 (12.8)	0.49 (16.7)	0.80 (24.4)	3.98 (265)
BDE-206	0.11	86 (87)	4.72 (176)	< LOQ	0.58 (19.1)	0.86 (28.9)	1.65 (57.3)	111 (3545)

BDE-207	0.12	98 (99)	4.08 (147)	< LOQ	1.18 (39.7)	1.75 (56.3)	2.82 (82.0)	88.7 (2842)
BDE-208	0.08	98 (99)	1.87 (67.5)	< LOQ	0.42 (13.3)	0.59 (19.3)	1.07 (34.0)	48.1 (1540)
BDE-209	0.47	95 (96)	117 (4444)	< LOQ	8.76 (287)	14.1 (440)	28.9 (1074)	3245 (104000)
PCB-11	1.21	77 (78)	3.00 (105)	< LOQ	1.56 (53.6)	2.66 (84.5)	3.96 (132)	15.1 (438)
PCB-14	0.56	0 (0)	—	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
PCB-28	< 0.01	99 (100)	0.03 (0.99)	0.00 (0.24)	0.01 (0.61)	0.02 (0.81)	0.04 (1.16)	0.21 (4.36)
PCB-52	< 0.01	99 (100)	0.01 (0.18)	0.00 (0.06)	0.00 (0.11)	0.00 (0.14)	0.01 (0.19)	0.05 (1.62)
PCB-77	0.06	82 (83)	0.10 (3.55)	< LOQ	0.06 (2.37)	0.08 (2.97)	0.12 (3.83)	0.38 (17.2)
PCB-81	0.03	19 (19)	0.03 (1.04)	< LOQ	< LOQ	< LOQ	< LOQ	0.07 (1.56)
PCB-101	< 0.01	99 (100)	0.01 (0.37)	0.00 (0.10)	0.01 (0.18)	0.01 (0.25)	0.01 (0.34)	0.19 (6.20)
PCB-105	< 4.65	99 (100)	20.2 (666)	4.65 (177)	11.6 (459)	17.7 (591)	23.8 (785)	79.0 (2067)
PCB-114	< 1.84	99 (100)	7.70 (251)	1.84 (54.8)	4.04 (159)	6.80 (224)	10.8 (330)	30.9 (810)
PCB-118	< 26.7	99 (100)	119 (3955)	26.7 (1044)	71.5 (2821)	104 (3619)	149 (4881)	335 (9635)
PCB-123	0.63	97 (98)	1.28 (42.5)	< LOQ	0.78 (28.7)	1.14 (37.6)	1.53 (55.1)	3.93 (110)
PCB-126	< 0.14	99 (100)	0.60 (20.1)	0.14 (4.40)	0.36 (14.1)	0.55 (18.1)	0.75 (23.5)	2.10 (58.5)
PCB-138	< 0.11	99 (100)	0.46 (15.2)	0.11 (3.93)	0.27 (10.1)	0.39 (14.0)	0.58 (18.2)	1.39 (35.6)
PCB-153	< 0.22	99 (100)	0.80 (26.3)	0.22 (5.76)	0.42 (17.6)	0.68 (23.3)	1.07 (34.7)	2.36 (70.0)
PCB-156	< 18.4	99 (100)	82.0 (2668)	18.4 (526)	39.8 (1633)	70.7 (2128)	114 (3573)	327 (8664)

PCB-157	< 2.77	99 (100)	12.1 (393)	2.77 (78.3)	5.74 (241)	9.98 (344)	17.4 (528)	43.1 (1143)
PCB-167	< 4.85	99 (100)	20.8 (688)	4.85 (157)	12.0 (447)	18.0 (662)	27.5 (877)	57.8 (1481)
PCB-169	0.03	98 (99)	0.38(12.6)	< LOQ	0.20 (7.88)	0.29 (10.9)	0.55 (15.2)	0.90 (34.1)
PCB-180	< 0.09	99 (100)	0.48 (15.7)	0.09 (2.69)	0.23 (8.76)	0.35 (13.2)	0.65 (21.3)	3.46 (91.8)
PCB-189	< 1.33	99 (100)	7.69 (251)	1.33 (36.1)	3.73 (133)	5.70 (219)	10.7 (351)	50.5 (1339)
2,3,7,8-TCDD	< 0.01	30 (30)	0.02 (0.80)	< LOQ	< LOQ	< LOQ	0.03 (1.06)	0.05 (1.71)
1,2,3,7,8-PeCDD	0.01	40 (40)	0.06 (2.14)	< LOQ	< LOQ	< LOQ	0.08 (2.73)	0.16 (4.33)
1,2,3,6,7,8-HxCDD	0.01	87 (88)	0.10 (3.26)	< LOQ	0.05 (2.22)	0.09 (2.94)	0.12 (3.99)	0.74 (14.2)
1,2,3,4,7,8-HxCDD	0.01	24 (24)	0.03 (0.95)	< LOQ	< LOQ	< LOQ	< LOQ	0.27 (5.19)
1,2,3,7,8,9-HxCDD	< 0.01	45 (45)	0.02 (0.75)	< LOQ	< LOQ	< LOQ	0.03 (0.94)	0.07 (1.70)
1,2,3,4,6,7,8-HpCDD	0.01	91 (92)	0.10 (3.26)	< LOQ	0.05 (1.96)	0.07 (2.62)	0.12 (3.89)	0.48 (14.7)
OCDD	0.01	98 (99)	0.65 (21.2)	< LOQ	0.33 (12.9)	0.48 (17.0)	0.85 (24.4)	3.44 (75.0)
2,3,7,8-TCDF	< 0.01	9 (9)	0.02 (0.78)	< LOQ	< LOQ	< LOQ	< LOQ	0.05 (1.50)
1,2,3,7,8-PeCDF	< 0.01	6 (6)	0.01 (0.51)	< LOQ	< LOQ	< LOQ	< LOQ	0.03 (0.95)
2,3,4,7,8-PeCDF	0.01	94 (95)	0.13 (4.21)	< LOQ	0.08 (3.01)	0.11 (3.80)	0.16 (5.15)	0.29 (10.8)
1,2,3,4,7,8-HxCDF	< 0.01	76 (77)	0.03 (1.14)	< LOQ	0.02 (0.78)	0.03 (1.01)	0.04 (1.24)	0.29 (5.52)
1,2,3,6,7,8-HxCDF	< 0.01	80 (81)	0.04 (1.18)	< LOQ	0.02 (0.72)	0.03 (1.03)	0.04 (1.41)	0.41 (7.86)
1,2,3,7,8,9-HxCDF	< 0.01	0 (0)	—	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

2,3,4,6,7,8-HxCDF	< 0.01	40 (40)	0.03 (0.92)	< LOQ	< LOQ	< LOQ	0.03 (0.86)	0.13 (6.69)
1,2,3,4,6,7,8-HpCDF	< 0.01	60 (61)	0.04 (1.39)	< LOQ	< LOQ	0.02 (0.84)	0.04 (1.27)	0.48 (23.8)
1,2,3,4,7,8,9-HpCDF	< 0.01	0 (0)	—	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
OCDF	< 0.01	1 (1)	—	< LOQ	< LOQ	< LOQ	< LOQ	0.02 (1.17)
2,3,7,8-TBDD	< 0.01	7 (7)	0.03 (1.13)	< LOQ	< LOQ	< LOQ	< LOQ	0.06 (2.15)
1,2,3,7,8-PeBDD	0.01	7 (7)	0.09 (2.64)	< LOQ	< LOQ	< LOQ	< LOQ	0.23 (8.11)
1,2,3,4,7,8-HxBDD	0.02	2 (2)	0.14 (3.15)	< LOQ	< LOQ	< LOQ	< LOQ	0.24 (4.94)
1,2,3,6,7,8-HxBDD	0.02	3 (3)	0.09 (2.24)	< LOQ	< LOQ	< LOQ	< LOQ	0.20 (4.12)
1,2,3,7,8,9-HxBDD	0.04	1 (1)	—	< LOQ	< LOQ	< LOQ	< LOQ	0.02 (1.17)
1,2,3,4,6,7,8-HpBDD	0.13	38 (38)	0.28 (9.24)	< LOQ	< LOQ	< LOQ	0.39 (11.9)	0.73 (18.0)
OBDD	0.33	3 (3)	2.13 (59.8)	< LOQ	< LOQ	< LOQ	< LOQ	4.49 (108)
2,3,7,8-TBDF	< 0.01	18 (18)	0.05 (1.50)	< LOQ	< LOQ	< LOQ	< LOQ	0.14 (4.53)
1,2,3,7,8-PeBDF	0.01	1 (1)	—	< LOQ	< LOQ	< LOQ	< LOQ	0.04 (0.99)
2,3,4,7,8-PeBDF	0.01	1 (1)	—	< LOQ	< LOQ	< LOQ	< LOQ	0.06 (3.22)
1,2,3,4,7,8-HxBDF	0.02	1 (1)	—	< LOQ	< LOQ	< LOQ	< LOQ	0.17 (3.33)
1,2,3,4,6,7,8-HpBDF	0.02	34 (34)	0.31 (12.2)	< LOQ	< LOQ	< LOQ	0.30 (9.01)	2.41 (88.2)
OBDF	1.22	4 (4)	31.5 (203)	< LOQ	< LOQ	< LOQ	< LOQ	69.1 (1337)
ΣPBDEs	—	99 (100)	157 (5753)	9.65 (511)	35.2 (1123)	50.2 (1731)	79.6 (2727)	3526 (112998)

ΣPCBs	—	99 (100)	276 (9090)	66.9 (2222)	159 (6238)	250 (8322)	365 (11211)	773 (20225)
ΣPCDD/Fs	—	99 (100)	1.08 (35.7)	0.00 (0.00)	0.61 (24.3)	0.83 (30.2)	1.31 (41.6)	4.34 (115)
ΣPBDD/Fs	—	99 (100)	1.58 (43.2)	0.00 (0.00)	0.00 (0.00)	0.14 (4.53)	0.38 (11.5)	69.88 (1352)

Table 4.6 WHO$_{2005}$-TEQ values of PCDD/Fs, PBDD/Fs, and dl-PCBs. Values are shown in pg/g lw.

	N (%)	Mean	SD	Min	25% percentile	Median	75% percentile	Max
PCB-77	82 (83)	0.0003	0.0002	< LOQ	0.0002	0.0003	0.0004	0.002
PCB-81	19 (19)	0.0002	0.0001	< LOQ	< LOQ	< LOQ	< LOQ	0.0008
PCB-126	99 (100)	2.01	0.87	0.44	1.41	1.81	2.35	5.85
PCB-169	98 (99)	0.37	0.19	< LOQ	0.24	0.33	0.45	1.02
PCB-105	99 (100)	0.02	0.01	0.005	0.01	0.02	0.02	0.06
PCB-114	99 (100)	0.008	0.004	0.002	0.005	0.007	0.01	0.02
PCB-118	99 (100)	0.12	0.05	0.03	0.08	0.11	0.15	0.29
PCB-123	97 (98)	0.001	0.0006	< LOQ	0.0008	0.001	0.002	0.003
PCB-156	99 (100)	0.08	0.04	0.02	0.05	0.06	0.11	0.26
PCB-157	99 (100)	0.01	0.006	0.002	0.007	0.01	0.02	0.03
PCB-167	99 (100)	0.02	0.009	0.005	0.01	0.02	0.03	0.04
PCB-189	99 (100)	0.008	0.005	0.001	0.004	0.007	0.01	0.04
2,3,7,8-TBDD	7 (7)	0.13	0.34	< LOQ	< LOQ	< LOQ	< LOQ	2.15

1,2,3,7,8-PeBDD	7 (7)	0.39	0.95	< LOQ	< LOQ	< LOQ	< LOQ	8.11
1,2,3,4,7,8-HxBDD	2 (2)	0.04	0.05	< LOQ	< LOQ	< LOQ	< LOQ	0.49
1,2,3,6,7,8-HxBDD	3 (3)	0.04	0.04	< LOQ	< LOQ	< LOQ	< LOQ	0.41
1,2,3,7,8,9-HxBDD	1 (1)	—	—	< LOQ	< LOQ	< LOQ	< LOQ	0.46
1,2,3,4,6,7,8-HpBDD	38 (38)	0.05	0.04	< LOQ	< LOQ	< LOQ	0.08	0.18
OBDD	3 (3)	0.002	0.004	< LOQ	< LOQ	< LOQ	< LOQ	0.03
2,3,7,8-TBDF	18 (18)	0.03	0.07	< LOQ	< LOQ	< LOQ	< LOQ	0.45
1,2,3,7,8-PeBDF	1 (1)	—	—	< LOQ	< LOQ	< LOQ	< LOQ	0.03
2,3,4,7,8-PeBDF	1 (1)	—	—	< LOQ	< LOQ	< LOQ	< LOQ	0.96
1,2,3,4,7,8-HxBDF	1 (1)	—	—	< LOQ	< LOQ	< LOQ	< LOQ	0.33
1,2,3,4,6,7,8-HpBDF	34 (34)	0.04	0.13	< LOQ	< LOQ	< LOQ	0.03	0.88
OBDF	4 (4)	0.01	0.05	< LOQ	< LOQ	< LOQ	< LOQ	0.40
2,3,7,8-TCDD	30 (30)	0.27	0.40	< LOQ	< LOQ	< LOQ	0.50	1.71
1,2,3,7,8-PeCDD	40 (40)	0.94	1.13	< LOQ	< LOQ	< LOQ	1.79	4.33
1,2,3,4,7,8-HxCDD	87 (88)	0.03	0.06	< LOQ	0.007	0.009	0.02	0.52
1,2,3,6,7,8-HxCDD	24 (24)	0.29	0.20	< LOQ	< LOQ	< LOQ	< LOQ	1.42
1,2,3,7,8,9-HxCDD	45 (45)	0.04	0.04	< LOQ	< LOQ	< LOQ	0.07	0.17
1,2,3,4,6,7,8-HpCDD	91 (92)	0.03	0.02	< LOQ	0.02	0.03	0.04	0.15

OCDD	98 (99)	0.006	0.004	< LOQ	0.004	0.005	0.007	0.02
2,3,7,8-TCDF	9 (9)	0.01	0.02	< LOQ	< LOQ	< LOQ	< LOQ	0.15
1,2,3,7,8-PeCDF	6 (6)	0.003	0.004	< LOQ	< LOQ	< LOQ	< LOQ	0.03
2,3,4,7,8-PeCDF	94 (95)	1.20	0.58	< LOQ	0.85	1.12	1.54	3.23
1,2,3,4,7,8-HxCDF	76 (77)	0.09	0.08	< LOQ	0.04	0.09	0.12	0.55
1,2,3,6,7,8-HxCDF	80 (81)	0.10	0.09	< LOQ	0.05	0.09	0.12	0.79
1,2,3,7,8,9-HxCDF	0 (0)	—	—	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
2,3,4,6,7,8-HxCDF	40 (40)	0.04	0.08	< LOQ	< LOQ	< LOQ	0.06	0.67
1,2,3,4,6,7,8-HpCDF	60 (61)	0.009	0.02	< LOQ	< LOQ	0.005	0.01	0.24
1,2,3,4,7,8,9-HpCDF	0 (0)	—	—	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
OCDF	1 (1)	—	—	< LOQ	< LOQ	< LOQ	< LOQ	0.0004
Σmono-ortho PCBs	99 (100)	2.38	0.99	0.54	1.74	2.20	2.78	6.57
Σnon-ortho PCBs	99 (100)	0.27	0.11	0.06	0.18	0.25	0.33	0.60
ΣPCBs	99 (100)	2.65	1.08	0.60	1.93	2.43	3.11	7.06
ΣPCDD/Fs	99 (100)	3.06	1.98	0.28	1.50	2.44	4.37	10.6
ΣPBDD/Fs	99 (100)	0.91	1.07	0.15	0.35	0.60	0.93	8.42
ΣPOPs	99 (100)	6.61	2.99	1.45	4.21	5.90	8.22	16.3

Table 4.7 Comparison of PBDE concentrations in human breast milk measured in different populations (ng/g lipid). Data are shown as median unless specified.

POPs	This study	US [63]	Beijing, China [208]	16 provinces, China [209]	Taiwan, China [210]	South Korea [211]	Indonesia [212]	Japan [213]	Greece [214]	Belgium [215]	UK [216]	Norway [217]
BDE-17	< LOQ	0.02	—	—	—	—	—	—	—	—	—	—
BDE-28	0.0293	1.3–2.0	0.094	0.22	0.03	—	0.03	0.04	< 0.10	< LOQ	—	0.106
BDE-47	0.204	7.7–31.5	0.066	0.25	0.22	0.54	0.30	0.68	0.48	0.16	1.92	1.031
BDE-66	0.0042	0.14–0.2	—	—	—	—	—	—	—	—	—	—
BDE-85	0.0093	0.3–0.5	—	—	—	—	—	—	—	—	—	—
BDE-99	0.0625	1.5–6.4	0.016	0.08	0.06	0.29	0.15	0.41	0.27	0.06	0.88	0.271
BDE-100	0.0543	0.5–5.7	0.016	0.06	0.08	0.12	0.11	0.38	0.19	0.06	—	0.257
BDE-153	0.377	1.1–8.0	0.247	0.5	0.63	—	0.22	0.31	0.30	0.29	1.01	0.497

BDE-154	0.00825	0.2–0.3	0.019	0.04	0.03	—	0.03	0.23	< 0.10	0.07	—	0.029
BDE-183	0.0284	0.06–0.2	0.027	0.32	0.05	—	0.09	—	< 0.10	< LOQ	0.05	—
BDE-196	0.0168	—	—	—	0.02	—	—	—	—	—	—	—
BDE-197	0.0731	—	—	—	0.12	—	0.21	—	—	—	—	—
BDE-203	0.0167	—	—	—	0.02	—	—	—	—	—	—	—
BDE-206	0.0289	—	—	—	0.05	—	< 0.05	—	—	—	—	—
BDE-207	0.0563	—	—	—	0.09	—	0.11	—	—	—	—	—
BDE-208	0.0193	—	—	—	0.04	—	—	—	—	—	—	—
BDE-209	0.44	1.41	2.2	—	0.69	—	< 1.3	—	—	0.65	0.52	—

Table 4.8 Comparison of PCB concentrations in human breast milk measured in different populations (ng/g lipid). Data are shown as median unless specified.

POPs	This study	US [63]	Canada [218]	16 provinces, China (mean) [209]	India (mean)[a] [219]	India (mean)[b] [219]	New Zealand (mean) [220]	Greece [214]	Norway [217]	Norway [221]	Slovak [222]	Belgium [215]
PCB-11	0.0845	—	—	—	—	—	—	—	—	—	—	—
PCB-14	< LOQ	—	—	—	—	—	—	—	—	—	—	—
PCB-28	0.00081	2.12–2.78	1.0	1.42	—	—	1.36956	—	—	—	—	< LOQ
PCB-52	0.00014	—	0.13	0.12	—	—	0.09692	—	—	—	—	< LOQ
PCB-77	0.00297	—	0.0012	—	0.16–0.63	0.018–0.024	0.00239	—	—	—	0.0031–0.0040	—
PCB-81	< LOQ	—	0.00098	—	0.075–0.16	0.013–0.014	0.00222	—	—	—	0.0014–0.0029	—
PCB-101	0.00025	—	0.17	0.1	—	—	0.11495	0.67	—	—	—	< LOQ

PCB-105	0.591	1.65–1.90	0.67	—	20–23	2.4–2.6	0.34872	1.3	1.35	1.4	0.616–0.971	0.7
PCB-114	0.224	—	0.18	—	2–2.8	0.34–0.35	0.06929	—	0.335	0.35	0.123–0.185	—
PCB-118	3.619	6.10–7.97	2.9	—	57–66	6.8–8.3	1.27967	4.6	5.945	6.82	4.213–5.078	3.7
PCB-123	0.0376	—	0.05	—	1.1–1.3	0.14–0.20	0.02190	—	—	—	0.039–0.0447	—
PCB-126	0.0181	0.012	0.0097	—	0.16–0.21	0.039–0.049	0.01009	—	—	—	0.0201–0.0325	—
PCB-138	0.014	0.04–0.18[a]	5.1	2.22	—	—	4.82487	13	19.412	20.3	—	13.5
PCB-153	0.0233	17–22.8	7.9	1.83	—	—	5.55128	24	30.9	34.7	—	16.8
PCB-156	2.128	2.04–5.39	0.93	—	8.2–12	1.8–1.9	0.68449	2.1	3.21	3.34	4.381–5.632	—
PCB-157	0.344	—	0.21	—	1.9–2.7	0.44–0.46	0.13521	—	0.67	0.61	0.4–0.495	—
PCB-167	0.662	—	0.23	—	3.2–4.0	0.55–0.69	0.18064	—	0.78	0.84	1.132–1.403	—
PCB-169	0.0109	0.0074	0.0045	—	0.014–0.024	0.011–0.012	0.00666	—	—	—	0.0098–0.0216	—
PCB-180	0.0132	7.78–10.6	4.0	0.9	—	—	2.91207	13	15.169	17.7	—	8.8
PCB-189	0.219	—	0.069	—	0.45–0.62	0.17–0.21	0.05285	—	0.23	0.25	0.478–0.748	—

Table 4.9 Comparison of dioxin concentrations in human breast milk measured in different populations (pg/g lipid). Data are shown as median unless specified.

POPs	This study	US [63]	Canada [218]	New Zealand (mean) [220]	Beijing, China [210]	India (mean)ᵃ [219]	India (mean)ᵇ [219]	Japan [223]	Solvak [222]	Spain [224]	Belgium [215]
2,3,7,8-TCDD	< LOQ	—	0.38	0.75	0.47	3.2–5.2	2.2–3.0	1.2	0.3–0.5	0.20–1.03	—
1,2,3,7,8-PeCDD	< LOQ	—	1.5	1.57	0.98	6.6–7.8	5.2–7.4	5.0	1.1–1.9	0.8–4	—
1,2,3,6,7,8-HxCDD	2.94	11	6.6	2.87	1.7	23–28	31–46	15.6	2.5–3.6	2.2–28.4	—
1,2,3,4,7,8-HxCDD	< LOQ	—	1.1	0.49	0.47	3.7–4.2	3.2–5.5	—	0.5–1.3	0.49–3.03	—
1,2,3,7,8,9-HxCDD	< LOQ	—	1.1	0.64	0.60	10–12	11–19	2.5	0.7–0.8	0.37–4.46	—
1,2,3,4,6,7,8-HpCDD	2.62	11	7.2	5.44	2.5	130–150	170–360	7.6	3.2–5.2	2.30–34	—
OCDD	17.0	70	28	30.53	33	370–400	540–980	60.7	15.5–21.6	15–143	—

2,3,7,8-TCDF	< LOQ	—	0.53	0.15	0.43	4.1–5.3	1.7–2.3	0.9	0.4–0.5	0.15–0.66	—
1,2,3,7,8-PeCDF	< LOQ	—	0.54	0.19	0.38	1.5–2.1	0.66–0.88	0.5	0.3–0.4	0.09–0.27	—
2,3,4,7,8-PeCDF	3.80	4	3	1.73	2.2	8.1–11	9.8–10	9.0	4.9–9.9	1.8–7.92	—
1,2,3,4,7,8-HxCDF	1.01	1.9	5.2	0.64	0.77	4–4.3	4.2–4.8	3.2	2.3–3.8	0.66–3.08	—
1,2,3,6,7,8-HxCDF	1.03	1.6	2.2	0.58	0.73	4.7–5.1	4.8–6.6	3.5	2–3.3	0.63–2.51	—
1,2,3,7,8,9-HxCDF	< LOQ	—	< LOD	0.36	0.12	0.039	< 0.6	—	0.05–0.1	0.05–0.99	—
2,3,4,6,7,8-HxCDF	< LOQ	—	0.82	0.38	0.44	1.6–1.8	2.3–2.4	2.3	0.6–1.5	0.14–0.31	—
1,2,3,4,6,7,8-HpCDF	0.84	2	5.4	1.72	0.76	13–14	18–30	1.6	0.7–1.3	0.05–2.17	—
1,2,3,4,7,8,9-HpCDF	< LOQ	—	1.2	0.43	0.16	0.17	0.52–0.63	—	0.1	0.07–0.17	—
OCDF	< LOQ	0.17	14	0.54	0.26	2	1–1.1	—	0.1–0.2	0.31–0.43	—
2,3,7,8-TBDD	< LOQ	—	—	—	—	—	—	—	—	—	0.05
1,2,3,7,8-PeBDD	< LOQ	—	—	—	—	—	—	—	—	—	< 0.07
1,2,3,4,7,8-HxBDD	< LOQ	—	—	—	—	—	—	—	—	—	< 0.07
1,2,3,6,7,8-HxBDD	< LOQ	—	—	—	—	—	—	—	—	—	—

1,2,3,7,8,9-HxBDD	< LOQ	—	—	—	—	—	—	—	—	—	< 0.04
1,2,3,4,6,7,8-HpBDD	< LOQ	—	—	—	—	—	—	—	—	—	—
OBDD	< LOQ	—	—	—	—	—	—	—	—	—	
2,3,7,8-TBDF	< LOQ	—	—	—	—	—	—	—	—	—	0.7
1,2,3,7,8-PeBDF	< LOQ	—	—	—	—	—	—	—	—	—	0.2
2,3,4,7,8-PeBDF	< LOQ	—	—	—	—	—	—	—	—	—	0.4
1,2,3,4,7,8-HxBDF	< LOQ	—	—	—	—	—	—	—	—	—	< 0.3
1,2,3,4,6,7,8-HpBDF	< LOQ	—	—	—	—	—	—	—	—	—	—
OBDF	< LOQ	—	—	—	—	—	—	—	—	—	—

[a]dumping site
[b]reference site
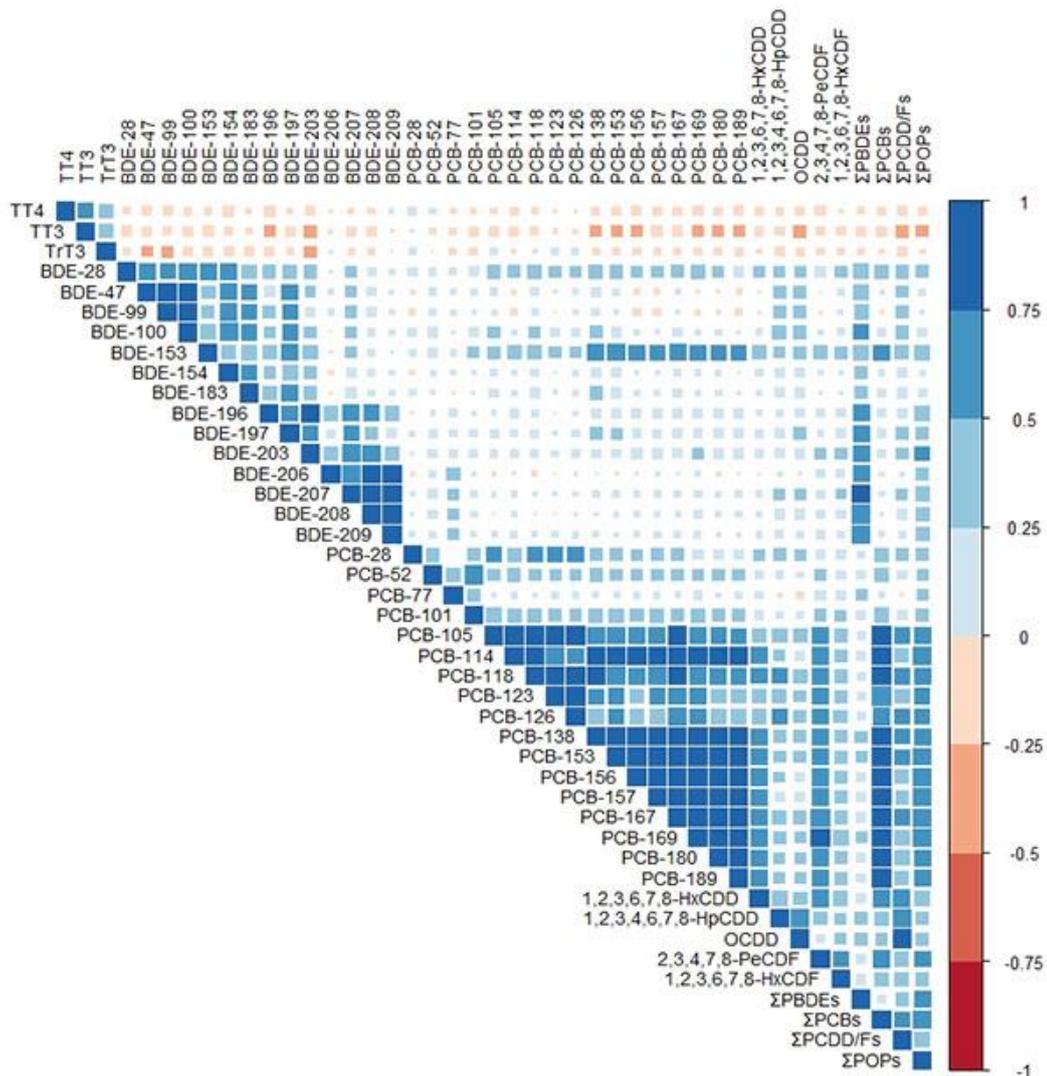
Fig. 4.6 Spearman's rank correlation of biomarkers measured in human breast milk. THs were expressed in ng/mL while POPs were expressed in pg/g lipid.

4.3.2 Population characteristics

Table 4.10 summarizes the demographic characteristics of all the participating women. The mean ± SD age of women 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; 62 (62.6%) received an education of > 12 years; 45 (45.5%) were nullipara; 84 (84.8%) had breast fed their infants; 89 (89.9%) were German citizens; 18 (18.2%) were vegetarians. The mean ± SD infant age at sampling was 114 ± 57 days.

As shown in Table 4.10, 4.11, 4.12, and 4.13, we observed significant correlations between demographic variables and biomarkers. For example, THs were correlated with maternal age,

ethnicity, and infant age at sampling.

Table 4.10 Correlations between demographic characteristics and THs in breast milk. Comparisons were performed using Welch t-test or analysis of variance (ANOVA).

	N (%)	TT_4 (ng/mL)	TT_3 (ng/mL)	TrT_3 (ng/mL)
Maternal age (y)				
≤29	15 (15.2)	0.75**	0.15**	0.03**
30–39	75 (75.8)	0.55	0.13	0.02
≥40	9 (9.1)	0.49	0.11	0.02
Maternal BMI (kg/m^2)				
< 24.9	66 (66.7)	0.58	0.13	0.02
25–29.9	19 (19.2)	0.57	0.14	0.02
≥30	11 (11.1)	0.58	0.13	0.02
Missing	3 (3.0)	0.47	0.12	0.03
Education				
< 12 years	37 (37.4)	0.62#	0.13	0.02
≥12 years	62 (62.6)	0.55	0.13	0.02
Parity				
1	45 (45.5)	0.60	0.13	0.02
2	46 (46.5)	0.56	0.13	0.02
≥3	8 (8.1)	0.55	0.14	0.02
Time of breastfeeding (month)				
Never	10 (10.1)	0.63	0.12	0.02
1–3	20 (20.2)	0.59	0.12	0.02
3–5	40 (40.4)	0.55	0.13	0.02
≥5	24 (24.2)	0.58	0.13	0.02
Missing	5 (5.1)	0.59	0.15	0.02
Ethnicity				
German	89 (89.9)	0.55**	0.13*	0.02

Non-German	10 (10.1)	0.80	0.15	0.03
Maternal smoking				
No	95 (95.0)	0.58	0.13	0.02
Yes	4 (4.0)	0.48	0.12	0.02
Vegetarian				
Yes	18 (18.2)	0.55	0.13	0.02
No	81 (81.8)	0.58	0.13	0.02
Infant age at sampling (d)				
< 60	19 (19.2)	0.68**	0.13	0.03**
60–120	37 (37.4)	0.54	0.13	0.02
120–180	28 (28.3)	0.50	0.13	0.02
≥180	14 (14.1)	0.64	0.13	0.02
Infant gender				
Female	47 (47.5)	0.61	0.13	0.02
Male	48 (48.5)	0.54	0.13	0.02
Missing	4 (4.0)	0.55	0.14	0.02

*$p < 0.05$; **$p < 0.01$. #$p < 0.10$

4.3.3 Single-pollutant model

As shown in Table 4.14 and Fig. 4.7, single-pollutant, crude models showed a statistically significant decrease in TT_4 with increasing exposure to BDE-99 [crude (c) β tertile 3 vs. 1: -0.16; 95% CI: -0.27 to -0.04; $p=0.01$], BDE-154 (c β tertile 3 vs. 1: -0.16; 95% CI: -0.28 to -0.05; $p=0.007$), BDE-169 (c β tertile 3 vs. 1: -0.13; 95% CI: -0.25 to -0.01; $p=0.03$), BDE-196 (c β tertile 3 vs. 1: -0.14; 95% CI: -0.26 to -0.02; $p=0.03$), BDE-203 (c β tertile 3 vs. 1: -0.13; 95% CI: -0.25 to -0.006; $p=0.04$), PCB-169 (c β tertile 3 vs. 1: -0.13; 95% CI: -0.25 to -0.01; $p=0.03$), and 1,2,3,6,7,8-HxCDD (c β tertile 3 vs. 1: -0.13; 95% CI: -0.25 to -0.01; $p=0.03$). After adjustment, TT_4 was negatively associated with BDE-99 [adjusted (adj) β tertile 2 vs. 1: -0.12; 95% CI: -0.24 to -0.01; $p=0.04$. adj β tertile 3 vs. 1: -0.16; 95% CI: -0.28 to -0.04; $p=0.01$], BDE-154 (adj β tertile 3 vs. 1: -0.14; 95% CI: -0.25 to -0.02; $p=0.02$), and BDE-196 (adj β tertile 3 vs. 1: -0.13; 95% CI: -0.25 to -0.003; $p=0.04$).

Table 4.11 Correlations between demographic characteristics and PBDEs in breast milk. Comparisons were performed using Welch t-test or analysis of variance (ANOVA). PBDE concentrations were expressed as pg/mL milk and pg/g lw (in the parentheses)

	N (%)	BDE-28	BD-E47	BDE-99	BDE-100	BDE-153	BDE-154	BDE-183	BDE-196	BDE-197	BDE-203	BDE-206	BDE-207	BDE-208	BDE-209
Maternal age (y)															
≤29	15	0.87	8.43	2.33	2.31	18.8	0.24	1.08	0.76	2.89	0.65	8.01	7.43	3.72	227
	(15.2)	(29.6)	(275)	(78.4)	(72.4)	(528)	(7.91)	(32.2)	(23.3)	(89.7)	(19.4)	(256)	(236)	(119)	(7293)
30–39	75	0.91	9.73	2.56	2.24	13.3	0.26	0.99	0.55	2.39	0.58	3.74	3.60	1.60	101
	(75.8)	(30.8)	(324)	(87.6)	(74.8)	(445)	(8.81)	(33.7)	(20.2)	(82.6)	(21.9)	(149)	(138)	(61.5)	(4120)
≥40	9 (9.1)	1.00	7.60	2.50	1.82	17.0	0.35	1.05	0.62	2.75	0.64	0.80	2.05	0.84	15.7
		(29.5)	(221)	(75.4)	(52.4)	(468)	(9.48)	(29.0)	(17.7)	(77.7)	(18.5)	(22.5)	(59.8)	(24.7)	(430)
Maternal BMI (kg/m^2)															
< 24.9	66	0.92	9.77	2.52	2.19	15.5	0.25	0.86#	0.54	2.45	0.59	2.83	3.09	1.36	75.0
	(66.7)	(30.9)	(312)	(82.7)	(70.4)	(494#)	(8.24)	(28.4#)	(18.9)	(81.6)	(21.6)	(113)	(117)	(51.6)	(3080)
25–29.9	19	0.90	9.16	2.74	2.32	11.6	0.31	1.14	0.77	2.62	0.67	9.30	7.80	3.81	269
	(19.2)	(32.7)	(358)	(109)	(86.8)	(399)	(10.9)	(40.3)	(28.6)	(94.4)	(24.4)	(339)	(278)	(135)	(9785)
≥30	11	0.81	8.46	2.44	2.45	12.4	0.26	1.22	0.47	2.42	0.47	1.53	2.42	1.02	33.1
	(11.1)	(23.7)	(240)	(70.4)	(70.6)	(350)	(8.05)	(36.8)	(14.3)	(71.1)	(13.2)	(44.2)	(69.7)	(29.9)	(1007)

Missing	3 (3.0)	1.16	4.29	1.43	1.15	17.1	0.29	2.62	0.84	3.21	0.75	9.15	7.09	3.34	239
		(33.9)	(125)	(41.8)	(33.4)	(497)	(8.40)	(76.2)	(25.0)	(92.5)	(22.0)	(279)	(215)	(101)	(7316)
Education (y)															
< 12	37	0.79#	8.30	2.31	2.08	13.8	0.26	1.02	0.60	2.52	0.54	4.39*	4.45	2.11	121#
	(37.4)	(26.5#)	(269)	(77.5)	(68.5)	(436)	(8.41)	(34.8)	(19.8)	(84.5)	(18.5)	(144*)	(147)	(69.3)	(3946#)
≥12	62	0.98	9.96	2.65	2.29	14.9	0.27	1.00	0.58	2.49	0.62	3.95	3.80	1.69	108
	(62.6)	(32.9)	(330)	(89.6)	(74.8)	(474)	(8.93)	(32.0)	(20.8)	(82.5)	(22.8)	(159)	(145)	(65.3)	(4456)
Parity															
1	45	0.97	8.85	2.26*	2.06	15.9	0.24	1.01	0.58	2.49	0.57	5.00	4.63	2.16	137
	(45.5)	(34.2)	(293)	(77.8#)	(68.7)	(506)	(8.41)	(32.8)	(19.5)	(81.6)	(19.0)	(164)	(154)	(71.4)	(4490)
2	46	0.85	9.85	2.70	2.29	13.2	0.28	1.02	0.59	2.50	0.62	3.68	3.71	1.68	102
	(46.5)	(27.0)	(324)	(90.9)	(74.4)	(420)	(9.06)	(33.6)	(21.6)	(84.8)	(24.1)	(163)	(150)	(69.0)	(4651)
≥3	8 (8.1)	0.90	9.15	2.99	2.62	13.9	0.27	0.95	0.62	2.56	0.56	1.67	2.68	1.09	33.7
		(30.3)	(286)	(92.4)	(81.9)	(430)	(8.74)	(31.2)	(18.9)	(83.0)	(17.0)	(38.6)	(71.9)	(28.2)	(779)
Time of breastfeeding (month)															
0	10	1.00	7.17	2.17	2.31	12.6	0.30	1.29	0.52	2.46	0.43	0.73*	1.65*	0.57*	13.2*
	(10.1)	(35.4)	(254)	(76.3)	(80.1)	(432)	(10.3)	(43.4)	(17.6#)	(84.7)	(15.1#)	(23.6*)	(55.8**)	(19.2*)	(410*)

1–3	20	0.87	8.22	2.30	2.19	17.6	0.25	1.05	0.88	3.03	0.83	12.5	10.3	5.10	359
	(20.2)	(29.8)	(288)	(83.8)	(71.4)	(519)	(8.65)	(34.0)	(29.2)	(99.6)	(27.2)	(410)	(337)	(166)	(11811)
3–5	40	0.93	10.6	2.69	2.41	14.2	0.27	1.02	0.53	2.37	0.57	2.27	2.62	1.12	60.9
	(40.4)	(32.4)	(354)	(92.8)	(81.1)	(446)	(9.17)	(34.2)	(20.8)	(81.9)	(23.8)	(126)	(123)	(55.4)	(3500)
≥5	24	0.85	9.58	2.68	1.99	12.0	0.25	0.86	0.49	2.29	0.50	2.38	2.76	1.17	56.1
	(24.2)	(24.7)	(280)	(80.3)	(58.7)	(401)	(7.55)	(26.5)	(14.7)	(70.6)	(15.1)	(68.9)	(80.8)	(33.2)	(1671)
Missing	5 (5.1)	1.07	6.79	1.96	1.56	20.4	0.23	0.88	0.45	2.55	0.56	0.56	1.55	0.54	10.3
		(37.1)	(239)	(68.3)	(57.2)	(682)	(8.14)	(30.3)	(15.5)	(85.7)	(18.1)	(19.3)	(50.9)	(17.5)	(367)
Ethnicity															
German	89	0.90	9.48	2.56	2.25	14.8	0.26	1.00	0.58	2.45	0.60	4.04	4.07	1.86	110
	(89.9)	(31.0)	(316)	(87.9*)	(74.8#)	(476**)	(8.92)	(33.5)	(20.8)	(83.3)	(21.9*)	(155)	(149)	(68.6)	(4300)
Non-German	10	0.99	8.08	2.14	1.88	11.4	0.27	1.04	0.61	2.98	0.51	4.76	3.78	1.72	135
	(10.1)	(26.7)	(225)	(60.1)	(51.0)	(316)	(7.09)	(28.6)	(17.1)	(82.2)	(14.7)	(138)	(114)	(50.5)	(3956)
Maternal smoking															
No	95	0.90	9.39	2.53	2.20	14.6	0.26	1.01	0.59	2.50	0.59	4.11#	4.03	1.85	114
	(95.0)	(30.7)	(313)	(86.6)	(73.3)	(468)	(8.86)	(33.6)	(20.7)	(84.4)	(21.4)	(157)	(148)	(67.9)	(4377)
Yes	4 (4.0)	1.21	8.19	2.28	2.43	12.7	0.27	0.88	0.60	2.52	0.76	4.18	4.31	1.96	80.0
		(26.0)	(172)	(48.4)	(50.6)	(277)	(5.86)	(18.9)	(12.7)	(56.0)	(16.0)	(84.6)	(88.7)	(40.2)	(1612)

Vegetarian														
Yes	18	1.02	8.37	2.12	2.33	19.9#	0.28	1.28	0.77*	3.06	0.93*	7.11*	6.16*	2.91*	202#
	(18.2)	(29.8)	(240)	(65.2)	(66.3)	(545)	(8.15)	(37.4)	(27.5#)	(91.5)	(35.8*)	(294*)	(248#)	(117#)	(8630#)
No	81	0.89	9.55	2.61	2.19	13.3	0.26	0.95	0.55	2.38	0.52	3.45	3.57	1.61	92.5
	(81.8)	(30.7)	(322)	(89.5)	(73.8)	(441)	(8.87)	(32.1)	(18.9)	(81.4)	(18.0)	(122)	(123)	(55.6)	(3295)
Infant age at sampling (d)														
< 60	19	0.94	9.21	2.42	2.08	15.2	0.24	0.88	0.86	2.72	0.76	13.6	10.9*	5.51#	390#
	(19.2)	(30.4)	(275)	(73.3)	(64.9)	(466)	(7.64)	(27.1)	(27.1)	(82.3)	(23.3)	(426)	(341)	(172)	(12276)
60–120	37	0.81	7.27	2.07	2.14	14.4	0.26	1.16	0.54	2.41	0.53	1.74	2.15	0.85	42.1
	(37.4)	(29.7)	(270)	(78.8)	(75.6)	(477)	(9.54)	(40.0)	(20.5)	(85.6)	(19.7)	(83.5)	(89.4)	(36.9)	(2112)
120–180	28	0.93	8.90	2.57	2.02	13.7	0.27	0.91	0.56	2.49	0.66	2.43	2.89	1.23	64.9
	(28.3)	(31.3)	(309)	(91.7)	(67.4)	(481)	(8.70)	(30.8)	(20.7)	(87.0)	(26.5)	(128)	(133)	(58.5)	(3651)
≥180	14	1.03	15.9	3.89	3.09	15.9	0.30	1.00	0.41	2.51	0.44	1.19	2.26	0.86	24.6
	(14.1)	(29.2)	(447)	(110)	(88.4)	(379)	(8.41)	(28.2)	(11.6)	(73.1)	(12.8)	(30.1)	(61.1)	(23.2)	(594)
Infant gender														
Female	47	0.93	8.38	2.39	2.42	14.5	0.26	1.07	0.62	2.63	0.60	3.96#	3.66	1.62#	104
	(47.5)	(29.6)	(259)	(75.9)	(73.8)	(437)	(8.25)	(33.8)	(20.5)	(81.8)	(19.3)	(138)	(123)	(54.7)	(3685)

Male	48	0.91	10.6	2.74	2.09	14.5	0.27	0.97	0.58	2.38	0.61	4.58	4.64	2.19	130
	(48.5)	(31.8)	(360)	(96.2)	(72.4)	(481)	(9.36)	(32.9)	(21.2)	(84.7)	(23.8)	(180)	(176)	(83.1)	(5168)
Missing	4 (4.0)	0.71	5.69	1.51	1.30	14.6	0.18	0.68	0.28	2.29	0.35	0.41	1.35	0.40	6.77
		(26.0)	(232)	(59.8)	(56.5)	(483)	(6.98)	(25.5)	(10.2)	(81.9)	(11.9)	(14.9)	(47.3)	(14.3)	(237)

*$p < 0.05$; **$p < 0.01$. #$p < 0.10$

Table 4.12 Correlations between demographic characteristics and PCBs in breast milk. Comparisons were performed using Welch t-test or analysis of variance (ANOVA). PBDE concentrations were expressed as pg/mL milk and pg/g lw (in the parentheses).

	N (%)	PCB-77	PCB-126	PCB-169	PCB-105	PCB-114	PCB-118	PCB-123	PCB-156	PCB-157	PCB-167	PCB-189	PCB-28	PCB-52	PCB-101	PCB-138	PCB-153	PCB-180
Maternal age (y)																		
≤29	15	0.10	0.59	0.34	21.8	8.29	121	1.31	87.5	13.2	20.8	7.10#	0.04	0.01	0.01	0.51	0.87	0.46#
	(15.2)	(2.93)	(18.5)	(10.6)	(671)	(251)	(3750)	(40.5)	(2634)	(396)	(637)	(213*)	(1.10)	(0.15)	(0.28)	(15.8)	(26.5)	(13.9#)
30–39	75	0.09	0.60	0.37	19.6	7.32	116	1.27	77.5	11.4	19.9	7.43	0.03	0.01	0.01	0.43	0.75	0.46
	(75.8)	(3.34)	(20.3)	(12.6)	(659)	(245)	(3943)	(42.9)	(2603)	(384)	(680)	(250)	(1.00)	(0.19)	(0.41)	(14.7)	(25.5)	(15.5)
≥40	9 (9.1)	0.07	0.66	0.48	22.4	9.82	140	1.20	111	15.7	27.6	10.9	0.02	0.00	0.01	0.62	1.12	0.68
		(2.32)	(20.6)	(14.6)	(719)	(298)	(4390)	(37.4)	(3272)	(467)	(844)	(324)	(0.74)	(0.14)	(0.26)	(18.1)	(33.0)	(20.4)
Maternal BMI (kg/m^2)																		

< 24.9	66 (66.7)	0.09 (3.28)	0.60 (20.2)	0.39 (13.3**)	20.1 (674)	8.01 (266*)	119 (4003)	1.29 (43.7#)	86.5# (2897**)	12.8 (426**)	21.4 (726*)	8.25* (277**)	0.03 (0.97)	0.01 (0.17)	0.01 (0.33)	0.46 (15.6)	0.82 (27.8*)	0.52* (17.3**)
25– 29.9	19 (19.2)	0.09 (3.02)	0.58 (20.6)	0.31 (10.5)	19.7 (680)	6.32 (209)	114 (3928)	1.14 (39.3)	64.6 (2076)	9.59 (311)	18.0 (611)	5.75 (185)	0.03 (0.97)	0.01 (0.22)	0.01 (0.29)	0.42 (14.0)	0.67 (22.4)	0.37 (11.9)
≥30	11 (11.1)	0.10 (3.00)	0.61 (18.0)	0.28 (8.21)	18.1 (523)	6.25 (180)	115 (3353)	1.15 (33.4)	65.3 (1840)	9.25 (266)	17.7 (509)	5.91 (162)	0.04 (1.02)	0.01 (0.23)	0.03 (0.79)	0.43 (12.2)	0.70 (20.0)	0.36 (10.2)
Missing	3 (3.0)	0.09 (2.66)	0.74 (21.5)	0.76 (22.0)	32.5 (939)	14.8 (428)	182 (5265)	1.88 (54.3)	154 (4431)	22.7 (652)	34.5 (998)	14.1 (408)	0.05 (1.48)	0.01 (0.20)	0.01 (0.34)	0.84 (24.2)	1.44 (41.6)	0.86 (24.7)
Education (y)																		
< 12	37 (37.4)	0.08 (2.72)	0.57 (18.9)	0.34 (10.9#)	20.8 (664)	7.58 (236#)	119 (3795)	1.27 (41.1)	76.3 (2351#)	11.4 (353#)	19.8 (628#)	6.77 (210#)	0.03 (1.03)	0.01 (0.19)	0.01 (0.29)	0.46 (14.4)	0.78 (24.2)	0.43 (13.4#)
≥12	62 (62.6)	0.10 (3.46)	0.62 (20.8)	0.40 (13.5)	19.8 (668)	7.76 (259)	120 (4050)	1.27 (42.5)	85.4 (2858)	12.5 (417)	21.3 (725)	8.24 (275)	0.03 (0.97)	0.01 (0.18)	0.01 (0.42)	0.46 (15.7)	0.81 (27.6)	0.51 (17.1)
Parity																		
1	45 (45.5)	0.10 (3.43*)	0.63 (21.3)	0.44* (15.1**)	22.0 (746#)	9.06# (302**)	130 (4406)	1.35 (45.7)	97.6# (3255**)	14.5* (485**)	23.1 (788*)	8.95 (299#)	0.03 (1.07#)	0.00 (0.16)	0.01 (0.33)	0.53 (17.9*)	0.92 (31.3*)	0.57 (19.0*)

2	46	0.09	0.57	0.32	18.0	6.50	107	1.17	68.9	10.1	18.5	6.62	0.03	0.01	0.01	0.40	0.68	0.40
	(46.5)	(3.10)	(18.7)	(10.3)	(588)	(208)	(3490#)	(38.6)	(2189)	(319)	(601)	(212)	(0.94)	(0.20)	(0.28)	(12.7)	(21.8)	(12.8)
≥3	8 (8.1)	0.07	0.66	0.33	22.3	6.89	133	1.34	69.6	9.98	20.5	6.73	0.03	0.01	0.04	0.46	0.76	0.44
		(2.27)	(20.6)	(10.3)	(670)	(211)	(4087)	(40.7)	(2124)	(307)	(630)	(206)	(0.84)	(0.24)	(1.12)	(14.3)	(23.9)	(13.4)
Time of breastfeeding (month)																		
0	10	0.08#	0.68	0.44	29.6#	11.3#	161	1.45	109	17.2	28.1	9.21	0.03	0.00	0.01	0.66#	1.10	0.61
	(10.1)	(2.90**)	(23.7)	(15.3)	(981*)	(370*)	(5441)	(48.6)	(3672)	(573#)	(963#)	(318)	(1.09)	(0.13)	(0.29)	(22.0*)	(37.2*)	(20.9)
1–3	20	0.11	0.61	0.38	18.5	7.02	111	1.25	74.4	11.1	19.8	6.85	0.03	0.00	0.01	0.43	0.74	0.42
	(20.2)	(3.72)	(20.1)	(12.1)	(621)	(227)	(3690)	(41.5)	(2363)	(351)	(653)	(218)	(0.99)	(0.15)	(0.27)	(14.0)	(23.8)	(13.2)
3–5	40	0.10	0.57	0.38	17.7	7.05	110	1.16	78.4	11.2	19.7	7.27	0.03	0.01	0.01	0.45	0.78	0.45
	(40.4)	(3.74)	(19.7)	(13.1)	(611)	(240)	(3781)	(40.2)	(2667)	(386)	(670)	(248)	(0.90)	(0.18)	(0.49)	(15.2)	(26.8)	(15.5)
≥5	24	0.07	0.61	0.36	21.8	7.97	123	1.36	84.8	12.5	20.2	8.68	0.04	0.01	0.01	0.45	0.77	0.55
	(24.2)	(2.20)	(18.9)	(11.0)	(661)	(242)	(3806)	(42.2)	(2553)	(373)	(632)	(261)	(1.17)	(0.26)	(0.33)	(13.5)	(23.4)	(16.3)
Missing	5 (5.1)	0.06	0.64	0.34	20.3	7.07	123	1.33	74.3	10.9	20.8	6.68	0.02	0.00	0.01	0.43	0.74	0.42
		(1.81)	(21.3)	(11.1)	(689)	(237)	(4139)	(44.9)	(2452)	(360)	(692)	(219)	(0.66)	(0.10)	(0.21)	(14.4)	(24.8)	(13.9)
Ethnicity																		

Germ an	89	0.09	0.60	0.38	19.4	7.32*	117	1.23	81.4	11.7	20.8	7.81	0.03**	0.01	0.01	0.46	0.80	0.49
	(89.9)	(3.23)	(20.4)	(12.8)	(660)	(246)	(3978)	(41.9)	(2703)	(392)	(702#)	(258#)	(0.93*)	(0.18)	(0.39)	(15.5)	(26.9)	(16.1#)
Non-Germ an	10	0.10	0.60	0.36	27.1	11.1	138	1.57	87.1	15.2	20.0	6.60	0.06	0.01	0.01	0.47	0.78	0.43
	(10.1)	(2.76)	(16.6)	(9.62)	(727)	(292)	(3747)	(42.8)	(2365)	(405)	(564)	(185)	(1.56)	(0.23)	(0.24)	(13.0)	(21.2)	(11.8)

Maternal smoking

No	95	0.09	0.60	0.37	19.8#	7.53#	118	1.25	81.0	11.9	20.6	7.62	0.03**	0.01**	0.01	0.46	0.79	0.48
	(95.0)	(3.24)	(20.3)	(12.5)	(667)	(250)	(3983)	(42.3)	(2678)	(393)	(694)	(252)	(0.95#)	(0.18#)	(0.38)	(15.3)	(26.5)	(15.8)
Yes	4 (4.0)	0.09	0.68	0.52	29.3	11.6	146	1.63	106	17.0	24.2	9.28	0.09	0.02	0.01	0.57	0.98	0.56
		(1.92)	(15.2)	(11.8)	(656)	(262)	(3274)	(36.4)	(2442)	(389)	(552)	(214)	(1.90)	(0.36)	(0.32)	(13.1)	(22.3)	(12.8)

Vegetarian

Yes	18	0.10	0.61	0.48	20.5	9.18	127	1.38	100	14.8	23.4	9.10	0.03	0.01	0.01	0.53	0.95	0.56
	(18.2)	(3.12)	(19.0)	(14.1)	(611)	(268)	(3820)	(42.2)	(2909)	(428)	(706)	(267)	(0.93)	(0.15)	(0.28)	(15.1)	(27.5)	(16.3)
No	81	0.09	0.60	0.35	20.1	7.37	118	1.24	78.0	11.5	20.2	7.38	0.03	0.01	0.01	0.45	0.76	0.46
	(81.8)	(3.20)	(20.3)	(12.1)	(679)	(247)	(3985)	(42.0)	(2615)	(386)	(685)	(247)	(1.00)	(0.19)	(0.39)	(15.2)	(26.1)	(15.6)

Infant age at sampling (d)

< 60	19	0.09*	0.64	0.38	23.4	8.88	130	1.43	85.5	13.3	22.0	7.44	0.04	0.00	0.01	0.49	0.83	0.46
	(19.2)	(2.84**)	(19.3)	(11.5**)	(705)	(265)	(3917)	(43.1)	(2541)	(391)	(664)	(221)	(1.17)	(0.14)	(0.23#)	(15.0)	(25.1)	(13.8)
60– 120	37	0.11	0.56	0.39	18.6	7.16	114	1.18	78.9	11.5	19.7	7.18	0.03	0.01	0.01	0.46	0.79	0.44
	(37.4)	(3.96)	(20.4)	(14.0)	(680)	(257)	(4115)	(42.9)	(2821)	(412)	(714)	(260)	(0.95)	(0.18)	(0.50)	(16.5)	(28.6)	(16.2)
120– 180	28	0.08	0.63	0.42	19.8	8.15	121	1.27	92.7	13.5	22.0	9.25	0.03	0.01	0.01	0.48	0.85	0.59
	(28.3)	(3.09)	(21.3)	(13.7)	(647)	(260)	(3985)	(41.5)	(2965)	(431)	(732)	(294)	(0.91)	(0.17)	(0.33)	(15.4)	(27.6)	(18.5)
≥180	14	0.06	0.58	0.26	20.2	6.24	116	1.29	65.3	9.14	19.7	6.51	0.03	0.01	0.01	0.41	0.68	0.41
	(14.1)	(1.88)	(17.8)	(7.80)	(610)	(188)	(3505)	(39.9)	(1901)	(270)	(580)	(190)	(1.00)	(0.27)	(0.34)	(11.8)	(19.9)	(12.0)
Infant gender																		
Female	47	0.09	0.65	0.42	22.2	8.65	130	1.39	94.7#	13.9#	22.9	9.01#	0.04*	0.01	0.01	0.51#	0.88#	0.57*
	(47.5)	(2.84)	(20.0)	(12.8)	(677)	(262)	(4008)	(42.2)	(2877)	(420)	(709)	(272)	(1.10)	(0.18)	(0.29#)	(15.7)	(27.3)	(17.1)
Male	48	0.09	0.56	0.35	18.5	6.92	111	1.17	71.3	10.6	18.9	6.59	0.03	0.01	0.01	0.43	0.73	0.41
	(48.5)	(3.53)	(20.2)	(12.4)	(656)	(241)	(3910)	(41.9)	(2483)	(371)	(667)	(233)	(0.91)	(0.19)	(0.47)	(14.7)	(25.6)	(14.5)
Missing	4 (4.0)	0.08	0.50	0.28	16.6	5.86	98.0	1.05	60.8	8.85	17.5	5.39	0.02	0.00	0.01	0.36	0.60	0.34
		(3.06)	(18.3)	(10.8)	(669)	(237)	(3863)	(41.0)	(2440)	(354)	(701)	(221)	(0.60)	(0.15)	(0.21)	(14.4)	(24.2)	(13.8)

Table 4.13 Correlations between demographic characteristics and PCDD/Fs, Σmono-ortho PCBs, Σnon-ortho PCBs, ΣPBDEs, and ΣPOPs in breast milk. Comparisons were performed using Welch t-test or analysis of variance (ANOVA). PBDE concentrations were expressed as pg/mL milk and pg/g lw (in the parentheses).

	N (%)	1,2,3,6,7,8-HxCDD	1,2,3,4,6,7,8-HpCDD	OCDD	2,3,4,7,8-PeCDF	1,2,3,6,7,8-HxCDF	ΣPBDE	ΣPCB	ΣPCDDF	ΣPOPs	Σmono-ortho PCBs	Σnon-ortho PCBs
Maternal age (y)												
≤29	15 (15.2)	0.06* (1.86*)	0.06 (1.91)	0.40 (13.1)	0.11 (3.45)	0.02 (0.66)	285 (9058)	284 (8682)	0.65* (20.9**)	569 (17761)	281 (8592)	1.03 (32.0)
30–39	75 (75.8)	0.09 (2.96)	0.10 (3.22)	0.67 (22.3)	0.12 (4.04)	0.03 (1.04)	144 (5597)	264 (8900)	1.01 (33.6)	408 (14531)	261 (8807)	1.06 (36.2)
≥40	9 (9.1)	0.14 (4.06)	0.09 (3.02)	0.79 (23.5)	0.15 (4.77)	0.03 (0.90)	54.7 (1535)	342 (10462)	1.21 (36.3)	398 (12033)	339 (10352)	1.21 (37.5)
Maternal BMI (kg/m^2)												
< 24.9	66 (66.7)	0.09 (3.06)	0.09 (2.99*)	0.61 (19.9)	0.12# (4.19**)	0.03# (1.01#)	118 (4510)	280 (9413*)	0.94 (31.2)	399 (13954)	277 (9314*)	1.08 (36.8#)
25–29.9	19 (19.2)	0.07 (2.38)	0.08 (2.98)	0.61 (22.5)	0.11 (3.80)	0.03 (1.03)	322 (11721)	242 (8125)	0.90 (32.6)	564 (19878)	239 (8041)	0.99 (34.1)
≥30	11 (11.1)	0.08 (2.07)	0.09 (2.53)	0.71 (21.2)	0.09 (2.52)	0.02 (0.50)	69.4 (2048)	241 (6939)	0.99 (28.8)	311 (9016)	238 (6866)	0.99 (29.2)

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missing	3 (3.0)	0.18 (5.34)	0.18 (5.35)	1.22 (36.1)	0.24 (6.88)	0.05 (1.49)	292 (8867)	461 (13313)	1.87 (55.2)	754 (22235)	456 (13175)	1.60 (46.1)
Education (y)												
< 12	37 (37.4)	0.09# (2.74#)	0.09 (2.93)	0.70 (23.5)	0.11 (3.61#)	0.04 (1.03)	164 (5349)	265 (8365#)	1.03 (33.8)	430 (13748#)	263 (8279#)	1.00 (32.5#)
≥12	62 (62.6)	0.09 (2.98)	0.09 (3.05)	0.60 (19.6)	0.12 (4.26)	0.03 (0.93)	153 (5993)	279 (9393)	0.93 (30.8)	433 (15417)	276 (9294)	1.11 (37.7)
Parity												
1	45 (45.5)	0.11 (3.44)	0.09 (3.11)	0.64 (21.8)	0.14 (4.66#)	0.04 (1.18)	184 (6021)	310 (10436*)	1.01 (34.2)	494 (16492#)	306 (10326*)	1.16 (39.9*)
2	46 (46.5)	0.08 (2.44)	0.08 (2.79)	0.62 (19.8)	0.10 (3.41)	0.02 (0.76)	145 (6143)	239 (7725)	0.90 (29.2)	385 (13897)	236 (7645)	0.98 (32.1)
≥3	8 (8.1)	0.07 (2.41)	0.12 (3.66)	0.76 (23.9)	0.12 (3.85)	0.03 (1.02)	73.7 (1996)	273 (8362)	1.10 (34.9)	348 (10393)	271 (8275)	1.06 (33.2)
Time of breastfeeding (month)												
0	10 (10.1)	0.09 (3.30)	0.07 (2.79)	0.67 (24.4)	0.16 (5.62)	0.03 (0.99)	46.4* (1557*)	370 (12489#)	1.02 (37.1)	417 (14083#)	366 (12366#)	1.20 (41.9)
1–3	20 (20.2)	0.09 (2.85)	0.08 (2.73)	0.56 (18.8)	0.12 (4.18)	0.03 (0.91)	424 (13914)	253 (8253)	0.88 (29.4)	677 (22197)	250 (8165)	1.10 (35.9)
3–5	40 (40.4)	0.10 (3.10)	0.09 (3.12)	0.63 (21.1)	0.11 (3.88)	0.04 (1.15)	102 (4980)	256 (8739)	0.97 (32.3)	359 (13750)	253 (8643)	1.04 (36.5)

≥5	24 (24.2)	0.08 (2.38)	0.11 (3.34)	0.74 (22.2)	0.11 (3.39)	0.02 (0.69)	93.9 (2833)	283 (8658)	1.05 (32.0)	378 (11522)	280 (8571)	1.04 (32.1)
Missing	5 (5.1)	0.09 (2.97)	0.06 (2.05)	0.54 (17.7)	0.13 (4.23)	0.03 (1.03)	49.3 (1696)	267 (8921)	0.85 (28.0)	317 (10645)	264 (8833)	1.04 (34.2)
Ethnicity												
German	89 (89.9)	0.09 (3.01*)	0.09 (3.14)	0.66 (22.0*)	0.12 (4.14*)	0.03 (1.00)	155 (5827)	270 (90778)	0.99 (33.3*)	426 (14937)	267 (8981)	1.07 (36.5)
Non-German	10 (10.1)	0.07 (1.82)	0.07 (1.79)	0.47 (12.5)	0.11 (2.89)	0.03 (0.73)	175 (5088)	309 (8405)	0.75 (19.8)	485 (13512)	307 (8328)	1.06 (28.9)
Maternal somking												
No	95 (95.0)	0.09 (2.90)	0.09 (3.07#)	0.64 (21.2)	0.12 (4.03)	0.03 (0.97)	158 (5889)	271 (9056)	0.96 (32.2)	430 (14977)	268 (8960)	1.06 (36.0)
Yes	4 (4.0)	0.12 (2.70)	0.08 (1.59)	0.71 (15.9)	0.17 (3.73)	0.04 (0.87)	122 (2508)	349 (7904)	1.12 (24.8)	472 (10438)	345 (7825)	1.29 (28.9)
Vegetarian												
Yes	18 (18.2)	0.15** (4.07*)	0.09 (2.86)	0.65 (19.0)	0.15# (4.47)	0.06* (1.49*)	256* (10436#)	308 (9147)	1.10 (31.9)	568* (19614#)	305 (9050)	1.19 (36.2)
No	81 (81.8)	0.08 (2.63)	0.09 (3.04)	0.64 (21.5)	0.11 (3.91)	0.02 (0.85)	134 (4712)	266 (8979)	0.94 (31.9)	401 (13722)	263 (8884)	1.04 (35.6)
Infant age at sampling												

< 60	19 (19.2)	0.09 (2.77)	0.09 (2.87)	0.71 (20.7)	0.12 (3.79)	0.03 (0.78)	455* (14293#)	295 (8837)	1.05 (31.0)	751* (23161#)	292 (8748)	1.12 (33.7#)
60–120	37 (37.4)	0.08 (2.87)	0.07 (2.75)	0.55 (20.8)	0.12 (4.32)	0.03 (0.90)	78.4 (3429)	261 (9403)	0.84 (31.6)	340 (12863)	258 (9302)	1.06 (38.3)
120–180	28 (28.3)	0.12 (3.48)	0.10 (3.42)	0.65 (22.0)	0.13 (4.43)	0.04 (1.33)	105 (5124)	291 (9456)	1.04 (34.7)	396 (14615)	288 (9355)	1.13 (38.0)
≥180	14 (14.1)	0.07 (2.11)	0.12 (3.22)	0.80 (21.2)	0.09 (2.92)	0.02 (0.74)	73.3 (1896)	246 (7357)	1.10 (30.2)	321 (9283)	244 (7284)	0.90 (27.5)
Infant gender												
Female	47 (47.5)	0.10 (2.83)	0.10* (3.14)	0.68 (20.9)	0.13# (4.18)	0.04 (1.01)	147 (5041)	306# (9364)	1.05 (32.0)	454 (14437)	303# (9267)	1.16 (35.6)
Male	48 (48.5)	0.09 (2.97)	0.08 (3.00)	0.62 (21.5)	0.11 (3.86)	0.03 (0.96)	177 (6820)	247 (8695)	0.92 (32.2)	425 (15547)	245 (8602)	1.00 (36.1)
Missing	4 (4.0)	0.07 (2.74)	0.04 (1.54)	0.45 (17.8)	0.10 (3.90)	0.02 (0.70)	36.6 (1307)	216 (8610)	0.68 (26.7)	254 (9944)	214 (8525)	0.85 (32.1)

Single pollutant, crude models revealed a decrease in TT_3 with increasing BDE-47 (c β tertile 3 vs. 1: -0.11; 95% CI: -0.21 to 0.00; p=0.04), BDE-100 (c β tertile 3 vs. 1: -0.10; 95% CI: -0.21 to 0.00; p=0.04), BDE-197 (c β tertile 2 vs. 1: -0.11; 95% CI: -0.21 to -0.01; p=0.04), BDE-203 (c β tertile 3 vs. 1: -0.17; 95% CI: -0.27 to -0.07; p=0.001), BDE-207 (c β tertile 3 vs. 1: -0.14; 95% CI: -0.24 to -0.04; p=0.01), BDE-208 (c β tertile 3 vs. 1: -0.12; 95% CI: -0.22 to -0.02; p=0.02), ΣPBDEs (c β tertile 3 vs. 1: -0.12; 95% CI: -0.22 to -0.02; p=0.02), PCB-101 (c β tertile 3 vs. 1: -0.11; 95% CI: -0.21 to -0.01; p=0.03), PCB-156 (c β tertile 2 vs. 1: -0.10; 95% CI: -0.21 to 0.00; p=0.04), PCB-169 (c β tertile 3 vs. 1: -0.14; 95% CI: -0.24 to -0.05; p=0.005), OCDD (c β tertile 3 vs. 1: -0.15; 95% CI: -0.25 to -0.06; p=0.002), ΣPCDD/Fs (c β tertile 3 vs. 1: -0.14; 95% CI: -0.24 to -0.04; p=0.01), ΣPOPs (c β tertile 3 vs. 1: -0.11; 95% CI: -0.21 to -0.01; p=0.03) (Table 4.14). After adjustment, TT_3 was negatively associated with BDE-47 (adj β tertile 3 vs. 1: -0.12; 95% CI: -0.22 to -0.02; p=0.02), BDE-99 (adj β tertile 3 vs. 1: -0.10; 95% CI: -0.21 to -0.002; p=0.046), BDE-100 (adj β tertile 3 vs. 1: -0.12; 95% CI: -0.22 to -0.02; p=0.02), BDE-197 (adj β tertile 3 vs. 1: -0.11; 95% CI: -0.21 to -0.01; p=0.04), BDE-203 (adj β tertile 3 vs. 1: -0.14; 95% CI: -0.24 to -0.03; p=0.01), BDE-207 (adj β tertile 3 vs. 1: -0.11; 95% CI: -0.20 to -0.01; p=0.04), and OCDD (adj β tertile 3 vs. 1: -0.10; 95% CI: -0.20 to -0.003; p=0.04) (Fig. 4.7).

Single-pollutant, crude models showed a decrease in TrT_3 with increasing BDE-47 (c β tertile 2 vs. 1: -0.22; 95% CI: -0.42 to -0.03; p=0.03. c β tertile 3 vs. 1: -0.23; 95% CI: -0.42 to -0.03; p=0.02), BDE-99 (c β tertile 3 vs. 1: -0.31; 95% CI: -0.50 to -0.11; p=0.002), BDE-100 (c β tertile 3 vs. 1: -0.22; 95% CI: -0.42 to -0.02; p=0.03), BDE-154 (c β tertile 3 vs. 1: -0.22; 95% CI: -0.41 to -0.02; p=0.03), BDE-183 (c β tertile 3 vs. 1: -0.24; 95% CI: -0.44 to -0.05; p=0.02), BDE-203 (c β tertile 3 vs. 1: -0.25; 95% CI: -0.45 to -0.05; p=0.01), 1,2,3,4,6,7,8-HpCDD (c β tertile 3 vs. 1: -0.24; 95% CI: -0.44 to -0.05; p=0.02) (Table 4.14). In adjusted single-pollutant models, TrT_3 was inversely associated with BDE-47 (adj β tertile 2 vs. 1: -0.24; 95% CI: -0.44 to -0.04; p=0.02), BDE-99 (adj β tertile 3 vs. 1: -0.27; 95% CI: -0.48 to -0.06; p=0.01), BDE-183 (adj β tertile 3 vs. 1: -0.21; 95% CI: -0.41 to -0.01; p=0.04), and BDE-203 (adj β tertile 3 vs. 1: -0.24; 95% CI: -0.46 to -0.02; p=0.03) (Fig. 4.7).
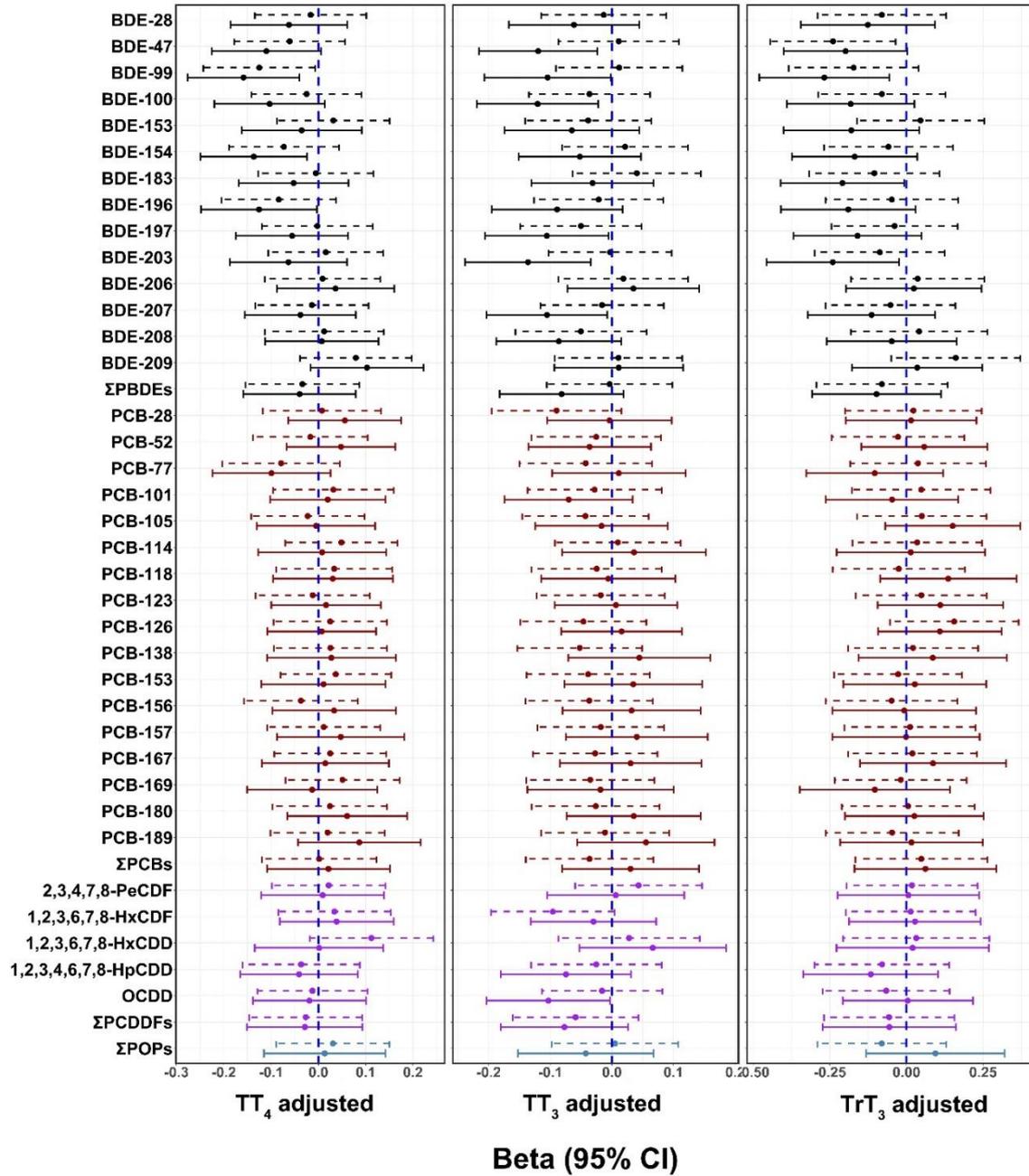
Fig. 4.7 Adjusted single pollutant models show the associations between exposure to tertiles of 36 POPs and THs in breast milk, single pollutant models. Dashed line represents the association of tertial 2 vs. 1 while the straight line represents the association of tertile 3 vs. 1. The estimated effects and corresponding confidence interval (95% CI) are shown by dots and error bars, respectively.

Table 4.14 Crude associations (β coefficient, 95% CI) between POPs (DF ≥ 80%) and THs in human breast milk, single pollutant models.

POPs		TT$_4$ β (95% CI)	p-value	TT$_3$ β (95% CI)	p-value	TrT$_3$ β (95% CI)	p-value
BDE-28	1	Reference		Reference		Reference	
	2	-0.05 (-0.17–0.07)	0.39	-0.02 (-0.12–0.09)	0.76	-0.12 (-0.32–0.08)	0.25
	3	-0.10 (-0.22–0.03)	0.12	-0.09 (-0.19–0.01)	0.09	-0.13 (-0.33–0.07)	0.19
BDE-47	1	Reference		Reference		Reference	
	2	-0.07 (-0.19–0.05)	0.23	-0.01 (-0.11–0.09)	0.82	-0.22 (-0.42–-0.03)	0.03
	3	-0.12 (-0.24–0.00)	0.05	-0.11 (-0.21–0.00)	0.04	-0.23 (-0.42–-0.03)	0.02
BDE-99	1	Reference		Reference		Reference	
	2	-0.12 (-0.24–0.00)	0.05	0.02 (-0.09–0.12)	0.75	-0.19 (-0.39–0.00)	0.05
	3	-0.16 (-0.27–-0.04)	0.01	-0.08 (-0.18–0.02)	0.13	-0.31 (-0.50–-0.11)	0.002
BDE-100	1	Reference		Reference		Reference	
	2	-0.06 (-0.18–0.06)	0.33	-0.06 (-0.16–0.05)	0.28	-0.13 (-0.33–0.07)	0.20
	3	-0.12 (-0.24–0.01)	0.06	-0.10 (-0.21–0.00)	0.04	-0.22 (-0.42–-0.02)	0.03
BDE-153	1	Reference		Reference		Reference	
	2	-0.01 (-0.13–0.11)	0.90	-0.05 (-0.15–0.05)	0.34	-0.02 (-0.22–0.18)	0.86
	3	-0.07 (-0.19–0.05)	0.26	-0.10 (-0.20–0.01)	0.06	-0.20 (-0.40–0.00)	0.05
BDE-154	1	Reference		Reference		Reference	

	2	-0.11 (-0.23–0.01)	0.07	-0.01 (-0.12–0.09)	0.78	-0.09 (-0.28–0.11)	0.39
	3	-0.16 (-0.28--0.05)	0.01	-0.07 (-0.17–0.03)	0.19	-0.22 (-0.41--0.02)	0.03
BDE-183	1	Reference		Reference		Reference	
	2	-0.03 (-0.15–0.10)	0.67	0.00 (-0.11–0.10)	0.95	-0.14 (-0.34–0.05)	0.15
	3	-0.06 (-0.19–0.06)	0.30	-0.05 (-0.15–0.06)	0.37	-0.24 (-0.44--0.05)	0.02
BDE-196	1	Reference		Reference		Reference	
	2	-0.11 (-0.23–0.01)	0.07	-0.04 (-0.14–0.06)	0.47	-0.14 (-0.34–0.06)	0.16
	3	-0.14 (-0.26--0.02)	0.03	-0.10 (-0.20–0.00)	0.05	-0.20 (-0.40–0.00)	0.05
BDE-197	1	Reference		Reference		Reference	
	2	-0.06 (-0.18–0.06)	0.35	-0.11 (-0.21--0.01)	0.04	-0.10 (-0.30–0.10)	0.31
	3	-0.05 (-0.18–0.07)	0.38	-0.09 (-0.19–0.01)	0.07	-0.16 (-0.36–0.04)	0.11
BDE-203	1	Reference		Reference		Reference	
	2	-0.02 (-0.14–0.10)	0.74	-0.02 (-0.12–0.08)	0.68	-0.13 (-0.32–0.07)	0.20
	3	-0.13 (-0.25--0.01)	0.04	-0.17 (-0.27--0.07)	0.001	-0.25 (-0.45--0.05)	0.01
BDE-206	1	Reference		Reference		Reference	
	2	-0.03 (-0.15–0.09)	0.62	0.00 (-0.10–0.11)	0.93	0.01 (-0.19–0.21)	0.93
	3	-0.05 (-0.17–0.08)	0.45	-0.02 (-0.12–0.09)	0.74	0.01 (-0.19–0.22)	0.89
BDE-207	1	Reference		Reference		Reference	

	2	-0.06 (-0.19–0.06)	0.29	-0.02 (-0.12–0.08)	0.65	-0.07 (-0.27–0.13)	0.48
	3	-0.10 (-0.22–0.03)	0.12	-0.14 (-0.24–-0.04)	0.01	-0.15 (-0.35–0.05)	0.14
BDE-208	1	Reference		Reference		Reference	
	2	-0.07 (-0.20–0.05)	0.23	-0.07 (-0.17–0.03)	0.18	-0.04 (-0.24–0.16)	0.71
	3	-0.07 (-0.19–0.06)	0.29	-0.12 (-0.22–-0.02)	0.02	-0.09 (-0.30–0.11)	0.36
BDE-209	1	Reference		Reference		Reference	
	2	0.06 (-0.06–0.19)	0.30	0.02 (-0.08–0.12)	0.69	0.15 (-0.05–0.35)	0.15
	3	0.03 (-0.09–0.16)	0.59	-0.02 (-0.12–0.08)	0.71	0.02 (-0.18–0.22)	0.87
ΣPBDEs	1	Reference		Reference		Reference	
	2	-0.11 (-0.23–0.01)	0.08	-0.07 (-0.17–0.04)	0.20	-0.19 (-0.39–0.01)	0.06
	3	-0.11 (-0.23–0.01)	0.07	-0.12 (-0.22–-0.02)	0.02	-0.13 (-0.33–0.07)	0.19
PCB-28	1	Reference		Reference		Reference	
	2	0.03 (-0.09–0.15)	0.65	-0.08 (-0.18–0.02)	0.76	-0.02 (-0.22–0.19)	0.87
	3	0.10 (-0.02–0.22)	0.10	0.01 (-0.09–0.11)	0.09	0.07 (-0.13–0.27)	0.49
PCB-52	1	Reference		Reference		Reference	
	2	0.02 (-0.10–0.14)	0.77	-0.04 (-0.14–0.06)	0.45	-0.01 (-0.21–0.19)	0.93
	3	0.05 (-0.07–0.17)	0.40	-0.04 (-0.14–0.06)	0.46	0.03 (-0.17–0.23)	0.78
PCB-77	1	Reference		Reference		Reference	

		Estimate (CI)	p	Estimate (CI)	p	Estimate (CI)	p
	2	-0.04 (-0.17–0.08)	0.47	-0.09 (-0.19–0.02)	0.10	0.01 (-0.19–0.21)	0.93
	3	-0.09 (-0.21–0.03)	0.14	-0.05 (-0.16–0.05)	0.31	-0.12 (-0.32–0.08)	0.23
PCB-101	1	Reference		Reference		Reference	
	2	-0.02 (-0.14–0.10)	0.72	-0.07 (-0.18–0.03)	0.15	-0.05 (-0.26–0.15)	0.60
	3	-0.01 (-0.14–0.11)	0.83	-0.11 (-0.21–-0.01)	0.03	-0.09 (-0.29–0.11)	0.37
PCB-105	1	Reference		Reference		Reference	
	2	-0.08 (-0.20–0.04)	0.18	-0.07 (-0.17–0.03)	0.19	-0.07 (-0.27–0.14)	0.52
	3	-0.07 (-0.19–0.06)	0.29	-0.08 (-0.18–0.03)	0.14	0.02 (-0.18–0.22)	0.82
PCB-114	1	Reference		Reference		Reference	
	2	0.00 (-0.12–0.12)	0.99	-0.02 (-0.13–0.08)	0.68	0.00 (-0.20–0.20)	0.98
	3	-0.07 (-0.19–0.06)	0.29	-0.07 (-0.17–0.04)	0.20	-0.07 (-0.27–0.13)	0.49
PCB-118	1	Reference		Reference		Reference	
	2	-0.05 (-0.18–0.07)	0.38	-0.06 (-0.17–0.04)	0.23	-0.11 (-0.31–0.09)	0.29
	3	-0.06 (-0.18–0.06)	0.32	-0.08 (-0.19–0.02)	0.11	-0.03 (-0.23–0.18)	0.80
PCB-123	1	Reference		Reference		Reference	
	2	0.03 (-0.09–0.15)	0.66	0.03 (-0.07–0.13)	0.56	0.08 (-0.12–0.29)	0.41
	3	-0.02 (-0.14–0.10)	0.72	-0.02 (-0.13–0.08)	0.68	0.06 (-0.14–0.26)	0.57
PCB-126	1	Reference		Reference		Reference	

	2	0.08 (-0.04–0.21)	0.17	0.01 (-0.09–0.11)	0.86	0.15 (-0.05–0.35)	0.13
	3	-0.02 (-0.14–0.10)	0.71	0.00 (-0.11–0.10)	0.96	0.06 (-0.14–0.26)	0.57
PCB-138	1	Reference		Reference		Reference	
	2	0.00 (-0.13–0.12)	0.94	-0.06 (-0.16–0.04)	0.24	-0.03 (-0.23–0.17)	0.78
	3	-0.06 (-0.18–0.06)	0.33	-0.08 (-0.18–0.03)	0.14	-0.08 (-0.28–0.12)	0.42
PCB-153	1	Reference		Reference		Reference	
	2	0.00 (-0.12–0.12)	0.96	-0.05 (-0.16–0.05)	0.30	-0.05 (-0.25–0.15)	0.62
	3	-0.09 (-0.21–0.04)	0.17	-0.09 (-0.19–0.01)	0.08	-0.11 (-0.31–0.10)	0.30
PCB-156	1	Reference		Reference		Reference	
	2	-0.10 (-0.23–0.02)	0.09	-0.10 (-0.21–0.00)	0.04	-0.09 (-0.29–0.11)	0.37
	3	-0.07 (-0.19–0.05)	0.25	-0.09 (-0.19–0.01)	0.07	-0.13 (-0.32–0.07)	0.22
PCB-157	1	Reference		Reference		Reference	
	2	-0.02 (-0.14–0.10)	0.73	-0.06 (-0.16–0.04)	0.23	0.00 (-0.20–0.20)	0.97
	3	-0.05 (-0.18–0.07)	0.39	-0.08 (-0.19–0.02)	0.11	-0.11 (-0.31–0.09)	0.26
PCB-167	1	Reference		Reference		Reference	
	2	-0.06 (-0.18–0.06)	0.32	-0.08 (-0.18–0.02)	0.12	-0.06 (-0.26–0.14)	0.56
	3	-0.10 (-0.22–0.02)	0.11	-0.09 (-0.19–0.01)	0.08	-0.08 (-0.28–0.12)	0.45
PCB-169	1	Reference		Reference		Reference	

	2	0.00 (-0.11–0.12)	0.94	-0.08 (-0.18–0.02)	0.11	-0.02 (-0.21–0.18)	0.11
	3	-0.13 (-0.25–-0.01)	0.03	-0.14 (-0.24–-0.05)	0.005	-0.18 (-0.38–0.02)	0.15
PCB-180	1	Reference		Reference		Reference	
	2	-0.06 (-0.19–0.06)	0.31	-0.10 (-0.20–0.00)	0.06	-0.11 (-0.31–0.09)	0.28
	3	-0.07 (-0.19–0.05)	0.26	-0.08 (-0.19–0.02)	0.10	-0.14 (-0.33–0.06)	0.18
PCB-189	1	Reference		Reference		Reference	
	2	-0.08 (-0.20–0.04)	0.19	-0.10 (-0.21–0.00)	0.05	-0.16 (-0.36–0.04)	0.11
	3	-0.06 (-0.18–0.06)	0.31	-0.08 (-0.18–0.02)	0.11	-0.15 (-0.34–0.05)	0.15
ΣPCBs	1	Reference		Reference		Reference	
	2	-0.03 (-0.15–0.10)	0.67	-0.06 (-0.16–0.04)	0.25	0.02 (-0.19–0.22)	0.87
	3	-0.07 (-0.19–0.05)	0.25	-0.08 (-0.18–0.03)	0.14	-0.06 (-0.27–0.14)	0.53
2,3,4,7,8-PeCDF	1	Reference		Reference		Reference	
	2	-0.04 (-0.16–0.08)	0.52	0.03 (-0.07–0.13)	0.57	-0.04 (-0.24–0.16)	0.67
	3	-0.08 (-0.20–0.05)	0.22	-0.08 (-0.18–0.03)	0.14	-0.11 (-0.32–0.09)	0.26
1,2,3,6,7,8-HxCDF	1	Reference		Reference		Reference	
	2	0.04 (-0.08–0.16)	0.50	-0.07 (-0.17–0.03)	0.18	0.00 (-0.20–0.20)	0.99
	3	0.00 (-0.13–0.12)	0.96	-0.06 (-0.16–0.05)	0.28	-0.04 (-0.24–0.16)	0.69
1,2,3,6,7,8-HxCDD	1	Reference		Reference		Reference	

		Estimate (95% CI)	p	Estimate (95% CI)	p	Estimate (95% CI)	p
	2	-0.04 (-0.16–0.08)	0.52	-0.08 (-0.18–0.02)	0.12	-0.11 (-0.31–0.09)	0.29
	3	-0.13 (-0.25–-0.01)	0.03	-0.09 (-0.19–0.01)	0.09	-0.15 (-0.35–0.05)	0.14
1,2,3,4,6,7,8-HpCDD	1	Reference		Reference		Reference	
	2	-0.05 (-0.17–0.07)	0.40	-0.04 (-0.14–0.07)	0.49	-0.12 (-0.32–0.08)	0.23
	3	-0.11 (-0.23–0.01)	0.08	-0.10 (-0.20–0.01)	0.07	-0.24 (-0.44–-0.05)	0.02
OCDD	1	Reference		Reference		Reference	
	2	-0.04 (-0.16–0.08)	0.55	-0.03 (-0.13–0.07)	0.59	-0.12 (-0.32–0.08)	0.23
	3	-0.10 (-0.22–0.02)	0.09	-0.15 (-0.25–-0.06)	0.002	-0.11 (-0.31–0.09)	0.29
ΣPCDDFs	1	Reference		Reference		Reference	
	2	-0.03 (-0.15–0.09)	0.57	-0.07 (-0.17–0.03)	0.18	-0.11 (-0.30–0.09)	0.29
	3	-0.11 (-0.23–0.01)	0.07	-0.14 (-0.24–-0.04)	0.01	-0.18 (-0.38–0.01)	0.07
ΣPOPs	1	Reference		Reference		Reference	
	2	0.01 (-0.11–0.13)	0.86	-0.01 (-0.11–0.09)	0.84	-0.10 (-0.30–0.10)	0.33
	3	-0.05 (-0.18–0.07)	0.39	-0.11 (-0.21–-0.01)	0.03	-0.01 (-0.21–0.19)	0.95

4.3.4 Multi-pollutant model

(1) Factor analysis

PCA was performed on the 36 POPs. As shown in Fig. 4.8. The first 3 PCs explained 35.2%, 14.3%, and 11.6% of the total variances, respectively. PC1 was mainly loaded with PCBs, PC2 and PC3 were mainly loaded with PBDEs (Fig. 4.8)

As shown in Fig. 4.9, the first 6 PCs had eigen values of > 1. These PCs were included in the same multiple linear regression as single pollutant models. As shown in Fig. 4.9, TT_4 was negatively associated with PC2 and PC6. TT_3 was inversely associated with PC1 and PC2. TrT_3 was inversely associated with PC2, PC3, and PC6.

Using PCA on the 36 POPs, we generated five factors that sufficiently accounted for the total variance inherent in the data. Table 4.15) presents the factor loading. Factor 1 was highly loaded with PCB-114, -138, -153, -156, -157, -167, -169, -180, -189, 1,2,3,6,7,8-HxCDD, and BDE-153; Factor 2 was highly loaded with BDE-196, -203, -206, -207, -208, and -209. Factor 3 was highly loaded with BDE-28, -47, -99, -100, -154, -183, and -197. Factor 4 was 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. Factor 5 was highly loaded with PCB-52, -77, and -101.
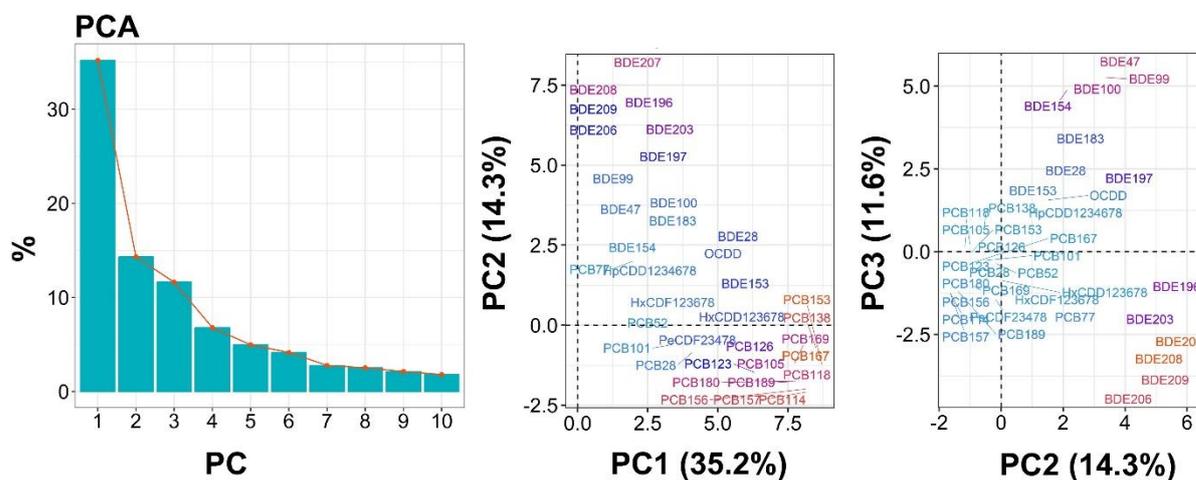
Fig. 4.8 Principal component analysis of the 36 POPs in human breast milk.
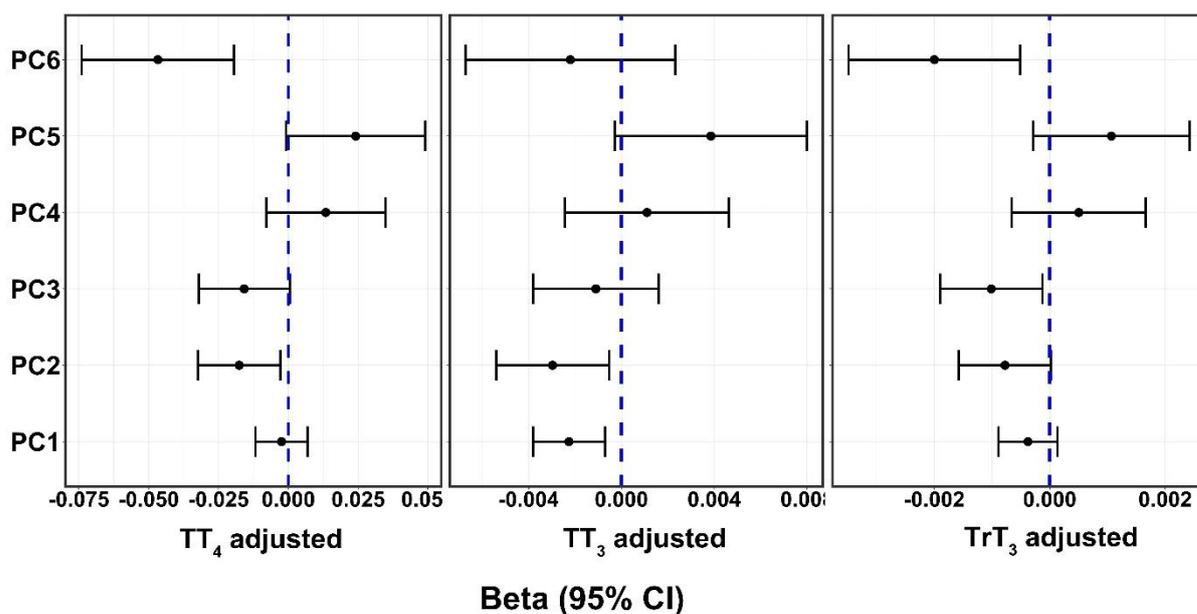
Fig. 4.9 Results of the Principal Component Regression (PCR). The estimated effects and corresponding confidence interval (95% CI) are shown by dots and error bars, respectively.

Table 4.15 Summary of the factor loading for five factors using principal component analysis with Varimax rotation.

	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	h2	u2	com
BDE-28	0.23	0.12	0.57	0.4	-0.01	0.55	0.448	2.3
BDE-47	-0.11	-0.03	0.88	0.14	0.02	0.81	0.189	1.1
BDE-99	-0.13	0.02	0.86	0.13	-0.05	0.78	0.217	1.1
BDE-100	0.01	-0.03	0.86	0.22	0.12	0.81	0.192	1.2
BDE-153	0.62	0.08	0.51	-0.01	-0.03	0.65	0.351	2
BDE-154	0.06	-0.08	0.78	-0.01	0.04	0.62	0.381	1
BDE-183	0.22	0.09	0.62	-0.12	0.04	0.46	0.54	1.4
BDE-196	0.17	0.75	0.27	-0.06	-0.05	0.67	0.334	1.4
BDE-197	0.21	0.38	0.69	-0.05	0	0.67	0.335	1.8
BDE-203	0.23	0.76	0.22	0.04	-0.03	0.68	0.321	1.4

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BDE-206	-0.08	0.89	-0.15	0.05	0.07	0.82	0.176	1.1
BDE-207	-0.05	0.96	0.1	0.09	0.02	0.94	0.063	1
BDE-208	-0.07	0.96	0.02	0.08	0.05	0.93	0.071	1
BDE-209	-0.06	0.89	-0.12	0.12	0.1	0.84	0.157	1.1
PCB-28	0.12	-0.01	-0.07	0.56	0.51	0.59	0.412	2.1
PCB-52	0.07	0	0.06	0.13	0.85	0.74	0.258	1.1
PCB-77	0.14	0.32	0	-0.05	0.49	0.37	0.633	1.9
PCB-101	0.19	-0.03	0.07	0.08	0.82	0.72	0.275	1.1
PCB-105	0.5	-0.09	0.11	0.67	0.3	0.81	0.187	2.4
PCB-114	0.82	-0.01	-0.03	0.41	0.24	0.9	0.104	1.7
PCB-118	0.58	-0.07	0.15	0.66	0.27	0.88	0.124	2.4
PCB-123	0.38	-0.04	-0.04	0.72	0.22	0.71	0.287	1.8
PCB-126	0.42	-0.03	0.07	0.74	0.16	0.75	0.248	1.7
PCB-138	0.82	-0.02	0.29	0.3	0.23	0.89	0.106	1.7
PCB-153	0.91	0	0.19	0.26	0.15	0.96	0.037	1.3
PCB-156	0.96	0.02	0	0.17	0.09	0.96	0.041	1.1
PCB-157	0.94	0	-0.01	0.21	0.12	0.94	0.062	1.1
PCB-167	0.85	0.01	0.13	0.42	0.09	0.93	0.068	1.5
PCB-169	0.84	0.06	0.05	0.24	0.12	0.79	0.213	1.2
PCB-180	0.95	0.01	0.05	0.12	0	0.92	0.084	1
PCB-189	0.94	0.03	0	0.11	-0.02	0.9	0.097	1
1,2,3,6,7,8-HxCDD	0.49	0.12	0.03	0.4	0.01	0.41	0.588	2.1

1,2,3,4,6,7,8-HpCDD	0.07	0.08	0.22	0.63	-0.1	0.47	0.528	1.4
OCDD	0.23	0.11	0.36	0.59	-0.2	0.59	0.415	2.4
2,3,4,7,8-PeCDF	0.46	0.14	-0.07	0.47	-0.1	0.47	0.533	2.3
1,2,3,6,7,8-HxCDF	0.25	0.21	0	0.45	0.07	0.31	0.687	2.1

As shown in Table 4.16, in the model that simultaneously included all five factors, exposure to tertile 3 compared with tertile 1 of the low-molecular weight PBDEs (factor 3) was associated with significant decrease in TT_4 (adj β: -0.16; 95% CI: -0.29 to -0.04; p=0.01) and TrT_3 (adj β: -0.29; 95% CI: -0.52 to -0.06; p=0.01). However, T_3 demonstrated a nonsignificant decrease (adj β: -0.10; 95% CI: -0.22 to 0.01; p=0.07) 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 to 0.00; p=0.047) and TrT_3 (adj β: -0.21; 95% CI: -0.41 to 0.00; p=0.047) were negatively associated with factor 3 in tertile 3, whereas nonsignificant association was found for TT_3 (adj β: -0.10; 95% CI: -0.19 to 0.00; p=0.06). Besides, in adjusted models, exposure to factor 4 showed an increase in TT_4 (adj β: 0.13; 95% CI: 0.00 to 0.26; p=0.04) and TrT_3 (adj β: 0.26; 95% CI: 0.03 to 0.49; p=0.03) in tertile 2 and nonsignificant association in tertile 3 compared with tertile 1.

(2) Hierarchical clustering

POPs were categorized into four groups using hierarchical clustering (Table 4.17 and Fig. 4.10). Model 1 includes PCB-114, -138, -153, -156, -157, -167, -169, -180, and -189; Model 2 includes 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; Model 3 includes BDE-28, 47, -99, -100, -153, -154, -183, and -197; Model 4 includes BDE-196, -203, -207, and -208. Grouped POPs were included in multiple linear regression models and demonstrated that TT_4 (adj β: -0.13; 95% CI: -0.23 to -0.02; p=0.03) and TrT_3 (adj β: -0.21; 95% CI: -0.42 to 0.00; p=0.04) were negatively associated with model 3 in the third tertile compared with tertile 1.
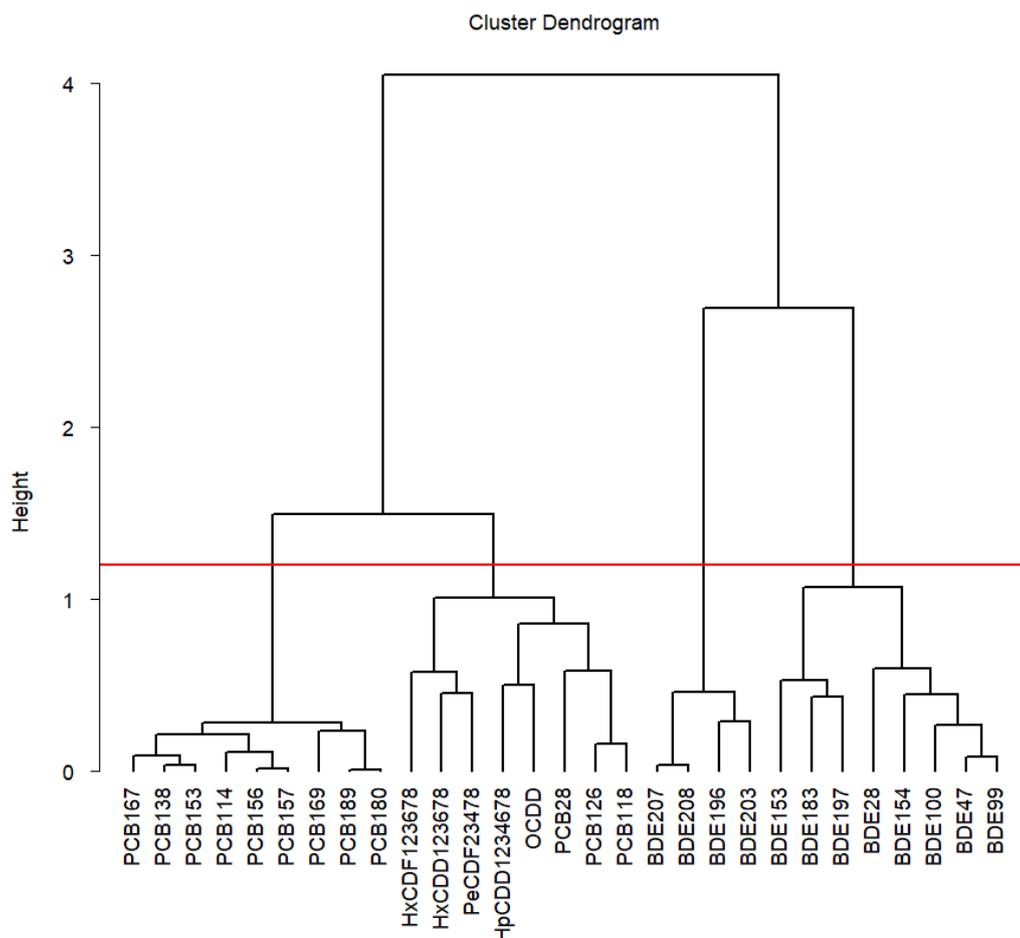
Fig. 4.10 Hierarchical clustering of POPs based on concentrations in 99 serum samples. The figure depicts the hierarchical structure obtained from the correlation between compounds (method: complete linkage). The horizontal red line represents the manually selected cut-off for the number of clusters.

(3) 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 4.18, 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.

Table 4.16 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[a]						
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[b]						
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[c]						
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[d]						
1	Reference	Reference	Reference	Reference	Reference	Reference

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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[e]						
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, country of origin, smoking, diet, and breastfeeding duration.
[a]Factor 1 loaded with PCB-114, -138, -153, -156, -157, -167, -169, -180, -189, 1,2,3,6,7,8-HxCDD, and BDE-153.
[b]Factor 2 loaded with BDE-196, -203, -206, -207, -208, and -209.
[c]Factor 3 loaded with BDE-28, -47, -99, -100, -154, -183, and -197.
[d]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.
[e]Factor 5 loaded with PCB-52, -77, and -101.

Table 4.17 Associations between POP and TH levels. POPs categorized based on hierarchical clustering.

	TT$_4$ β (95% CI)	TT$_3$ β (95% CI)	TrT$_3$ β (95% CI)
Model 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)
Model 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)
Model 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)*
Model 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, country of origin, smoking, diet, and breastfeeding duration.
Model 1 includes PCB-114, -138, -153, -156, -157, -167, -169, -180, and -189.
Model 2 includes 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.
Model 3 includes BDE-28, 47, -99, -100, -153, -154, -183, and -197.
Model 4 includes BDE-196, -203, -207, and -208.

Table 4.18 Associations between exposure to tertiles of five factors from principal component analysis and TH levels based on single- and multiple-factor models. All models were adjusted for maternal age, BMI, education level, parity, country of origin, smoking, diet, and breastfeeding duration.

	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[a]						
1	Reference	Reference	Reference	Reference	Reference	Reference
2	0.03 (-0.10–0.15)	0.04 (-0.07–0.15)	0.02 (-0.20–0.24)	0.02 (-0.10–0.14)	0.05 (-0.06–0.16)	-0.01 (-0.23–0.21)
3	0.07 (-0.07–0.22)	0.08 (-0.05–0.20)	0.00 (-0.25–0.26)	0.08 (-0.07–0.23)	0.09 (-0.05–0.22)	-0.06 (-0.32–0.21)
Factor 2[b]						
1	Reference	Reference	Reference	Reference	Reference	Reference
2	-0.01 (-0.14–0.13)	-0.01 (-0.12–0.10)	0.05 (-0.17–0.28)	0.01 (-0.14–0.15)	-0.01 (-0.13–0.12)	0.15 (-0.10–0.40)
3	0.00 (-0.13–0.14)	-0.04 (-0.16–0.07)	-0.05 (-0.28–0.18)	0.06 (-0.08–0.20)	-0.01 (-0.14–0.11)	0.05 (-0.20–0.30)
Factor 3[c]						
1	Reference	Reference	Reference	Reference	Reference	Reference

2	-0.04 (-0.16–0.09)	-0.02 (-0.13–0.08)	-0.12 (-0.33–0.09)	-0.07 (-0.20–0.05)	-0.04 (-0.16–0.07)	-0.22 (-0.44–0.01)
3	-0.12 (-0.24–0.00)*	-0.09 (-0.20–0.01)	-0.21 (-0.41–0.00)*	-0.16 (-0.29–-0.03)*	-0.10 (-0.22–0.01)	-0.30 (-0.53–-0.07)*

Factor 4[d]

1	Reference	Reference	Reference	Reference	Reference	Reference
2	0.10 (-0.03–0.24)	0.04 (-0.07–0.16)	0.20 (-0.02–0.43)	0.15 (0.01–0.29)*	0.06 (-0.06–0.18)	0.26 (0.02–0.51)*
3	-0.02 (-0.14–0.10)	-0.04 (-0.15–0.07)	0.08 (-0.13–0.30)	-0.01 (-0.14–0.11)	-0.03 (-0.14–0.08)	0.11 (-0.11–0.33)

Factor 5[e]

1	Reference	Reference	Reference	Reference	Reference	Reference
2	-0.06 (-0.19–0.06)	-0.07 (-0.18–0.04)	-0.02 (-0.24–0.20)	-0.03 (-0.16–0.10)	-0.05 (-0.16–0.06)	0.02 (-0.21–0.24)
3	0.03 (-0.09–0.16)	0.00 (-0.10–0.11)	0.08 (-0.14–0.29)	0.01 (-0.11–0.14)	-0.01 (-0.12–0.10)	0.06 (-0.16–0.28)

4.4 Discussion

Previous study proved the transfer of THs from blood to breast milk [44]. 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 4.4) [44]. Another study found higher levels of TT_4 and TT_3 in pooled milk sample from patients with thyroid-related diseases using chemiluminescence immunoassay [207]. However, our results might be more reliable and accuracy since RIA method is prone to nonspecific interferences.

Due to the strong limitations in Germany, the POP levels have decreased for > 40% compared with previous results [201]. POP exposures in this population is also lower compared with other regions. For example, BDE-47 reported in this study were lower than those reported in the US (7.7–31.5 ng/g lw) [63] and Canada (20.5 ng/g lw) [72]. Based on the serum/milk participation ratio of POPs [62], the median serum levels of PCB-153, BDE-99 and OCDD of this population are estimated to be 19.1, 59.4, and 417 pg/g lw, respectively, which are lower than those reported in Norway (PCB-153: 0.16 ng/g lw) [225], the US (BDE-99: 4.0 ng/g lw) [226] and the Netherland (OCDD: 2050 ng/g lw) [227]. Therefore, our study can represent a low exposure population.

4.4.1 Associations of PBDEs with THs

We observed the highest thyroid-disrupting potencies for PBDEs among the POPs examined. Single pollutant models revealed inverse associations of THs with PBDEs such as BDE-47, -99, -100, -154, -183, -196, -197, -203, and -207. Similar results were obtained using factor analysis and hierarchical clustering, which proved an association of PBDEs (including BDE-28, -47, -99, -100, -154, -183, and -197) with depressed TT_4 and TrT_3. These findings are consistent with animal and epidemiologic studies, which support a negative relationship between PBDE exposure and THs [72-75]. Putative mechanisms underlying this observation include the interference of PBDEs with TH transport and metabolism. For example, *in vitro* studies demonstrated that lower-brominated OH-PBDEs are structurally resemble to THs and can competitively bind with thyroid hormone receptor (THR) [68, 69]. Animal studies also

showed enhanced TH metabolism following PBDE exposure [228].

In contrast, others observed positive or nonsignificant associations [81, 82]. The inconsistence is probably the results of random error given the intraindividual variability in THs, uncontrolled bias, specimen type, and determination methods. For example, Stapleton *et* al. [81] and Zota *et* al. [82] employed serum samples from women during pregnancy when marked fluctuations in HPT axis homeostasis occur [83]. Our samples were collected post-partum when HPT axis tends to be more stable. Another possibility is that the relationship between PBDEs and THs may vary by exposure level. THs act at quite low concentrations (free serum level: 8–20 ng/L [84]) while low-dose effects and non-monotonic responses are remarkably common in studies of EDC [85]. For example, Abdelouahab *et* al. observed significant decrease in TT_4 and TT_3 in lambs following low-dose exposure of BDE-47 [86]. A meta-analysis suggested that the relationship between THs and PBDEs might be negative when PBDEs < 30 ng/g, no correlation when PBDEs are 30–100 ng/g, and positive when PBDEs > 100 ng/g [87]. This is consistent with our findings because the estimated serum median level of PBDEs of this population are ≤ 11.0 ng/g.

4.4.2 Associations of PCBs, PCDD/Fs, and PBDD/Fs with THs

A substantial body of animal and epidemiologic studies have reported decreased THs with increasing exposure of PCBs, despite the literature is inconsistent [76-78]. We observed inverse associations of PCB-101, -156, -169, 1,2,3,6,7,8-HxCDD, OCDD, and 1,2,3,4,6,7,8-HpCDD with THs (TT_4 and TT_3) in single-pollutant unadjusted models. After adjustment, only the association of OCDD with TT_3 remained. In multipollutant approach, we observed positive associations of TT_4 and TrT_3 with Factor 4, which was mainly loaded with PCBs and PCDD/Fs. This finding might be due to the non-linear relationship between PCBs and THs. Langer *et* al. reported negative associations between PCBs and THs at low exposure levels, but positive relations at high exposure levels [229]. This report was consistent with our finding considering of the low exposure level of this population.

PBDD/Fs are brominated dioxins found as impurities of PBDEs and formed during the incineration and degradation of brominated chemicals [230]. Previous studies also observed low detection rates of PBDD/Fs in human breast milk from China and Ireland [231, 232].

Therefore, the thyroid-disrupting properties of these chemicals were not assessed here.

4.4.3 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.

4.5 Conclusion

To conclude, the findings suggest that low-level exposure to POPs, in particular PBDEs, might be associated with TH alterations in human breast milk. The results highlight the challenges of evaluating the thyroid-disrupting effects of POPs due to the complexity of exposure mixtures, bias, specimen selection, and the sensitivity of the thyroid system. Regarding the critical role of THs to metabolism, bone remodeling, cardiac function and mental status, associations of THs with POPs are of great public health significance.

Chapter 5 Association of placental thyroid hormones with gestational diabetes mellitus

Abstract

GDM is the symptom of glucose intolerance that is first detected during pregnancy. GDM may lead to serious adverse outcomes and increases the long-term risks of type 2 diabetes mellitus. The incidences of GDM and type 2 diabetes have been steadily increasing throughout the world. Recent studies found that maternal thyroid dysfunction, especially in early pregnancy, may be associated with GDM. This is of great importance because THs of maternal origin are critical for the proper fetal development, especially during early pregnancy. Even minor changes in maternal TH circulation can lead to various adverse outcomes. In this chapter, we firstly developed an LC-MS/MS method for the determination of THs (T_4, T_3, rT_3, $3,3'$-T_2, and $3,5$-T_2) in placenta. One hundred and seventeen placenta samples including 32 samples from women with GDM and 85 samples from healthy women were derived from a Chinese birth cohort. T_4, T_3, rT_3 and T_2 were measured with mean concentrations of 14.3, 0.39, 2.35, and 0.40 ng/g fresh weight, respectively. The results were in good agreement with previous studies using immunoassay and LC-Q-TOF-MS. The correlations between various demographical characteristics and THs and GDM were assessed using analysis of variance and Chi-square test. Maternal age, pregnancy BMI and parity were correlated with the odds of GDM. These variables were therefore included as cofounders. Multiple logistic regression was adopted for the analysis of the association between placental THs and GDM. However, no significant associations between THs and the odds of GDM were observed in this study, probably due to the small number of cases. This study provides insight into the association of GDM with the transplacental passage of THs.

Author contribution:

Zhong-Min Li was involved in the study design, data analysis, and data interpretation. Yan Wu collected the human placenta samples and the demographical characteristics. Meri De Angelis reviewed the results. Heqing Shen and Karl-Werner Schramm were involved in the

study design, sample collection, and data interpretation.

5.1 Introduction

In this chapter, we firstly developed an HPLC-QqQ-MS method for the determination of THs in human placenta. The methods were validated and modified for TH analysis in human placenta, mouse placenta, and mouse plasma. Samples were collected from a Chinese birth cohort study. The associations between THs and GDM were analyzed using Pearson's Chi-square test.

5.2 Materials and methods

5.2.1 Sample collection

The placenta samples were obtained from a birth cohort study performed in Xiamen Maternity and Child Care Hospital (Xiamen, China). The GDM screening tests consisted of a 50 g 1-hour glucose test (glucose challenge test) and a further oral glucose tolerance test (OGTT) based on the World Health Organization (WHO) criteria. Venous blood was taken 60 min after the ingestion of 200 mL of 25% glucose solution. Mothers, with a record of ≥ 7.8 mmol/L glucose at the initial screening were invited to undergo a 75 g 2-hour OGTT. Diabetes is defined as fasting serum glucose ≥ 7.0 mmol/L and/or 2-hour serum glucose ≥ 11.1 mmol/L, and impaired glucose tolerance (IGT) is defined as 2-hour serum glucose ≥ 7.8 and < 11.1 mmol/L and fasting serum glucose < 7.0 mmol/L. Women who had confirmed either diabetes and/or IGT are regarded as the GDM cases [233].

Placentas were collected at birth by the midwives and kept frozen in polyethylene tubes at -80 ℃. Upon analysis, the samples were defrosted, 100 mg sample was mechanically homogenized and aliquoted into a 15-mL centrifuge tube. In total, 117 samples were derived from mothers with (32) and without (85) GDM.

5.2.2 Determination of THs in placenta using LC-MS/MS

Placenta samples were processed with the same method as described in section 2.5. Detailed information about the hormones, sample preparation, extraction procedure, and reagents have been described earlier [37] and section 2.2.1.

The chromatographic optimization was based on previous method (chapter 2) with some modifications. The chromatographic separation was performed with a Nexera X2 UHPLC system (Shimadzu, Japan) equipped with an Agilent Poroshell 120 EC-C18 column (3 mm×150 mm, 2.7 μm, Agilent technologies, USA). Table 5.1 shows the optimized MS/MS parameters.

The method was evaluated by the method detection limits (MLODs), method quantification limits (MLOQs), accuracy (spike-recoveries) and precisions (intra-day and inter-day variances). Mthods for calculating these parameters are outlined in Section 2.3.

5.2.3 Statistics

T_4, T_3, rT_3, and 3,3'-T_2 were included in the statistical analyses. The THs were normally distributed. Normality was confirmed using Kolmogorov-Smirnov test. We used Spearman's rank correlations to evaluate the interrelationship of THs. Analysis of variance (ANOVA) and chi-square test were used to examine associations between demographic characteristics and TH concentrations. Adjusted and unadjusted logistic regressions were used to compare placental TH concentrations in cases with GDM and controls.

Potential confounders considered for inclusion in the models were parameters that probably influence THs, i.e., maternal age, gestational age, parity (nulliparous vs. one or more live birth), maternal pre-pregnant body mass index (BMI), smoking during pregnancy (yes vs. no), employment status (three categories: unemployed, employed, and other), education level (three categories: high school education or lower, associate degree, and bachelor or higher), infant gender, birth weight, and birth length. Final models included variables that were loosely associated with the THs ($p < 0.20$) in bivariate analyses.

All statistical analyses were conducted using R (version 3.4.2; R Foundation for Statistical Computing, Vienna, Austria). A p-value < 0.05 was considered significant, and $p < 0.10$ was considered as a tendency of association.

Table 5.1 Optimized MS/MS parameters and MRM transitions in positive-ion mode for the determination of thyroid hormones in human placenta.

	$^{13}C_6$-T_4	T_4	$^{13}C_6$-T_3	T_3	$^{13}C_6$-rT_3	rT_3	$^{13}C_6$-3,3'-T_2	3,3'-T_2	3,5-T_2
DP	26	25	26	26	26	26	27	27	27
EP	10	10	9	9	9	9	9	9	9
CE	37	36	32	32	32	32	27	27	27
CXP	37	35	27	27	27	27	22	22	22
MRM									
$[M+H]^+\rightarrow[M+H-HCOOH]^+$	783.5→737.4	777.5→731.4	657.6→611.6	651.6→605.6	657.6→611.6	651.6→605.6	531.6→485.6	525.6→479.6	525.6→479.6
$[M+H]^+\rightarrow[M+H-IOH]^+$		777.5→633.4		651.6→507.6		651.6→507.6		525.6→381.6	525.6→381.6

Abbreviations: DP: Declustering potential, EP: Entrance potential, CE: Collision energy, CXP: Collisioin cell exit potential.

5.3 Results and discussion

5.3.1 HPLC-QqQ-MS method development

As shown in Fig. 5.1, after optimization, T_4, T_3, rT_3, $3,3'$-T_2, and $3,5$-T_2 can be found, indicating improved sensitivity of this method.

As shown in Table 5.2, The MDLs were between 0.002 and 0.004 ng/g. The MQLs were in the range of 0.007–0.010 ng/g. The spike-recoveries were between 61.1% and 108%, with a CV of 0.2–11.0%. The intra-day and inter-day CVs were 0.40–12.7% and 1.49–9.61%, respectively. The presence of $3,5$-T_2 in human placenta was tracked for the first time. These results proved the high sensitivity and selectivity of this method.
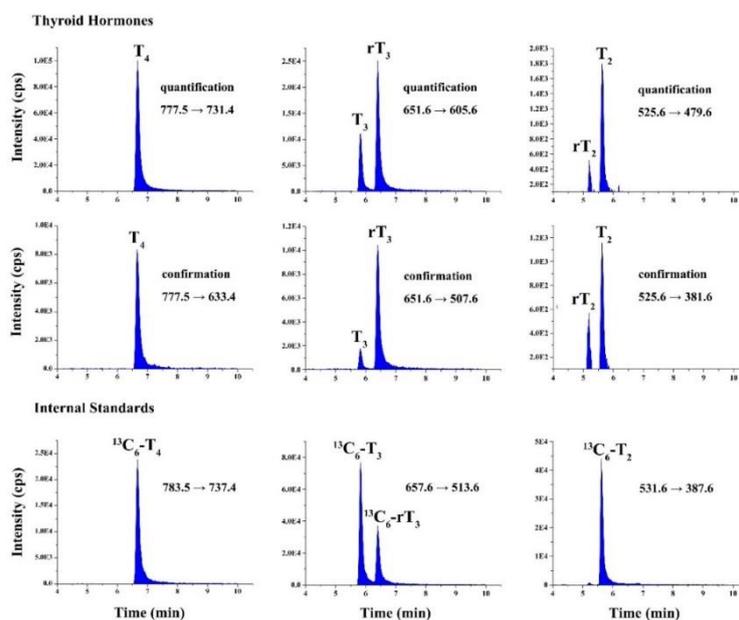

Fig. 5.1 MRM chromatograms of thyroid hormones detected in the human placenta: $[M + H]^+$ $\rightarrow$ $[M + H–HCOOH]^+$ transition for quantification and $[M + H]^+ \rightarrow [M + H–IOH]^+$ transition for confirmation. $3,5$-T_2 was not quantified in all human placenta samples (S/N < 10).

5.3.2 Association of placental THs with GDM

Table 5.3 summarizes the characteristics of all the participating women and newborns. Eighty-one (68.4%) women were ≤ 30 years old; forty-four (37.6%) were employed; forty (34.2%) had an education level of high school or lower; fifteen (12.8%) had a BMI value of > 24; sixty-eight (58.1%) were nullipara; all the babies (100%) were delivered at over 37 weeks

of gestation (full term). Maternal age, employment status, prepregnant BMI, and parity were correlated with GDM, and were thus included in multiple logistic regression as cofounders.

T_4, T_3, rT_3, and $3,3'$-T_2 were measured in all samples, while the detection frequency of $3,5$-T_2 was < 50% and therefore was not included in analysis. The mean concentration of T_4 was 28.8 ng/g fresh weight (fw) (range: 14.8–50.4 ng/g fw). The mean concentration of rT_3 was 2.37 ng/g fw (range: 0.31–5.57 ng/g fw). The mean level of T_3 was 0.40 ng/g fw (range: 0.01–1.30 ng/g fw). The mean concentration of $3,3'$-T_2 was 0.40 ng/g fw (range: 0.20–1.44 ng/g fw). THs were not correlated with any of the studied characteristics (Table 5.3).

Table 5.2 Method validation parameters of the optimized method for analyzing THs in human placenta.

	T_4	T_3	rT_3	$3,3'$-T_2	$3,5$-T_2
MDL (ng/g)	0.002	0.002	0.002	0.002	0.004
MQL (ng/g)	0.007	0.007	0.008	0.008	0.010
Spike-recovery (%), n=3					
1.5 (ng/g)	100 ± 5	114 ± 5	104 ± 8	108 ± 7	91.3 ± 11.0
15 (ng/g)	98.2 ± 2.4	113 ± 1	105 ± 2	104 ± 2	61.1 ± 0.2
30 (ng/g)	102 ± 2	110 ± 3	106 ± 4	103 ± 3	76.0 ± 6
Intra-day variation (%), n=3					
1.5 (ng/g)	0.40	3.74	12.1	5.93	11.7
15 (ng/g)	1.14	1.26	1.24	2.37	12.7
30 (ng/g)	1.27	2.66	2.94	2.94	7.89
Inter-day variation (%), n=6					
1.5 (ng/g)	3.13	1.69	9.61	9.00	8.73
15 (ng/g)	1.60	5.55	5.52	3.78	5.36
30 (ng/g)	1.64	4.18	6.21	6.54	1.49

Abbreviations: MDL: method detection limit, MQL: method quantification limit.

Table 5.3 Comparison of the clinical characteristics and THs across the study population. A Pearson's Chi-square test was used to evaluate the statistical significance of the correlations between GDM and population characteristics. ANOVA was used to assess the statistical significance of the correlations between THs and population characteristics.

Characteristics	N (%)	GDM (%)	p	Measures of THs in placenta (mean (SD))			
				T_4 (ng/g fw)	T_3 (ng/g fw)	rT_3 (ng/g fw)	$3,3'$-T_2 (ng/g fw)
Mean (SD)				28.8 (7.6)	0.40 (0.31)	2.37 (1.05)	0.40 (0.19)
Median (minimum, maximum)				28.0	0.34	2.22	0.35
				(14.8, 50.4)	(0.01, 1.30)	(0.31, 5.57)	(0.20, 1.44)
Maternal characteristics							
GDM	32 (27.4%)	32 (100%)	—	29.0 (6.9)	0.44 (0.32)	2.56 (1.18)	0.43 (0.20)
Non-GDM	85 (72.6%)	0 (0%)		28.8 (7.9)	0.38 (0.30)	2.29 (0.99)	0.39 (0.18)
Age (years)			0.012				
< 26	27 (23.7%)	2 (7.41%)		27.9 (8.5)	0.34 (0.34)	2.12 (0.93)	0.37 (0.17)
26-27	25 (21.9%)	8 (32.0%)		29.7 (8.6)	0.40 (0.28)	2.35 (1.14)	0.40 (0.24)
28-30	26 (22.8%)	6 (23.1%)		28.9 (6.9)	0.44 (0.30)	2.23 (0.82)	0.40 (0.14)
≥31	36 (31.6%)	16 (44.4%)		28.7 (6.9)	0.41 (0.32)	2.61 (1.19)#	0.42 (0.20)
Employment status			0.086				
Unemployed	43 (36.8%)	10 (23.3%)		29.2 (8.5)	0.43 (0.32)	2.45 (1.16)	0.42 (0.22)

Employed	44 (37.6%)	17 (38.6%)		27.1 (6.6)	0.35 (0.32)	2.25 (1.06)	0.38 (0.18)
Other	30 (25.6%)	5 (16.7%)		30.8 (7.2)	0.42 (0.29)	2.42 (0.88)	0.39 (0.14)
Education level			0.368				
High school or lower	40 (34.2%)	12 (30%)		29.4 (7.3)	0.47 (0.36)	2.41 (1.11)	0.44 (0.20)
Associate degree	37 (31.6%)	7 (18.9%)		28.8 (7.4)	0.34 (0.27)	2.44 (0.87)	0.36 (0.11)
Bachelor or higher	40 (34.2%)	13 (32.5%)		28.3 (8.1)	0.38 (0.26)	2.26 (1.15)	0.39 (0.22)
Pregnancy BMI (kg m^{-2})			< 0.01				
< 18.5	25 (21.4%)	1 (4.0%)		31.1 (9.4)	0.37 (0.34)	2.12 (0.86)	0.35 (0.14)
18.5-23.9	77 (65.8%)	22 (28.6%)		28.5 (7.0)	0.40 (0.31)	2.46 (1.13)	0.42 (0.21)
≥24	15 (12.8 %)	9 (60.0%)		26.5 (6.2)#	0.41 (0.27)	2.29 (0.85)	0.40 (0.15)
Gestational age (days)			0.371				
259-269	11 (9.4%)	4 (36.4%)		26.7 (5.9)	0.36 (0.21)	2.35 (0.46)	0.38 (0.15)
269-279	56 (47.9%)	12 (21.4%)		29.2 (8.2)	0.37 (0.31)	2.38 (1.23)	0.40 (0.21)
279-289	50 (42.7 %)	16 (32.0%)		28.9 (7.3)	0.44 (0.33)	2.35 (0.93)	0.41 (0.17)
Parity			0.010				
Primiparous	68 (58.1%)	12 (17.6%)		29.6 (7.8)	0.40 (0.31)	2.30 (0.97)	0.39 (0.19)
Multiparous	49 (41.9%)	20 (40.8%)		27.7 (7.2)	0.39 (0.31)	2.46 (1.15)	0.40 (0.19)
Infant characteristics							

Gender			0.970				
Male	60 (51.3%)	17 (28.3%)		29.2 (8.1)	0.39 (0.31)	2.36 (1.12)	0.40 (0.21)
Female	57 (48.7%)	15 (26.3%)		28.4 (7.1)	0.40 (0.30)	2.37 (0.97)	0.40 (0.16)
Birth weight (kg)			0.245				
< 3	20 (17.1%)	5 (25.0%)		29.1 (7.5)	0.40 (0.31)	2.25 (0.79)	0.38 (0.15)
3-4	92 (78.6%)	24 (26.1%)		28.9 (7.6)	0.40 (0.31)	2.36 (1.06)	0.40 (0.19)
≥4	5 (4.27%)	3 (60.0%)		25.7 (8.1)	0.37 (0.31)	2.88 (1.71)	0.45 (0.18)
Birth length (cm)			0.617				
≤50	100 (85.5%)	26 (26.0%)		28.7 (7.5)	0.40 (0.31)	2.34 (1.02)	0.39 (0.19)
> 50	17 (14.5%)	6 (35.3%)		29.8 (8.2)	0.41 (0.28)	2.50 (1.22)	0.43 (0.14)

Abbreviations: GDM: gestational diabetes mellitus; BMI: body mass index; SD: standard deviation; fw: fresh weight.

Table 5.4 GDM risk analysis of patients with different thyroid hormone levels in pregnancy.

	GDM N (+/-)	Incidence (%)	cOR (95% CI)	p	aOR (95% CI)	p
T_4 (ng/g fw)						
14.7-	5/24	17.2%	Reference		Reference	
24.0-	12/17	41.4%	4.14 (0.90, 22.3)	0.079#	5.52 (0.82, 46.8)	0.094#
28.0-	7/22	24.1%	2.18 (0.26, 22.8)	0.490	1.37 (0.10, 22.8)	0.816
33.7-	8/22	26.9%	3.16 (0.13, 93.9)	0.486	1.43 (0.02, 108)	0.865
p for trend		0.258				
T_3 (ng/g fw)						
0.002-	8/21	27.6%	Reference		Reference	
0.139-	5/24	17.2%	0.68 (0.16, 2.67)	0.581	0.55 (0.10, 2.79)	0.479
0.336-	8/21	27.6%	1.74 (0.29, 11.6)	0.554	1.17 (0.14, 10.4)	0.885
0.619-	11/19	36.7%	4.73 (0.22, 122)	0.327	2.29 (0.06, 108)	0.660
p for trend		0.423				
rT_3 (ng/g fw)						
0.31-	7/22	24.1%	Reference		Reference	
1.57-	8/21	27.6%	0.98 (0.27, 3.62)	0.972	0.71 (0.16, 3.10)	0.651
2.21-	6/23	20.7%	0.54 (0.10, 2.84)	0.462	0.23 (0.03, 1.68)	0.155

2.91-	11/19	36.7%	0.78 (0.06, 9.30)	0.847	0.28 (0.01, 5.27)	0.404
p for trend		0.550				
3,3'-T$_2$ (ng/g fw)						
0.203-	7/22	24.1%	Reference		Reference	
0.280-	8/21	27.6%	1.18 (0.35, 4.05)	0.784	1.04 (0.23, 4.60)	0.957
0.349-	6/23	20.7%	0.80 (0.20, 3.13)	0.747	0.70 (0.13, 3.77)	0.677
0.473-	11/19	36.7%	1.71 (0.26, 11.9)	0.575	0.75 (0.07, 7.66)	0.807
p for trend		0.550				

cOR: crude odds ratio.

aOR: adjusted for age, prepregnancy BMI, employment status, other THs.

95%CI: 95% confidence interval.

As shown in Table 5.4 & 5.5, Multiple logistic regression was used to analyze the association between TH levels and GDM. THs were modeled both as continuous variable and in quantiles. However, none of the THs showed a significant difference between the case and control groups.

Table 5.5 GDM risk analysis of patients with different thyroid hormone levels in pregnancy

THs (ng/g fw)	cOR (95% CI)	p	aOR (95% CI)	p
T_4	1.00 (0.90, 1.06)	0.861	1.00 (0.93, 1.08)	0.914
T_3	1.80 (0.48, 6.58)	0.374	1.54 (0.09, 26.2)	0.764
rT_3	1.27 (0.87, 1.87)	0.211	1.24 (0.68, 2.27)	0.483
$3,3'-T_2$	2.83 (0.33, 23.8)	0.324	0.57 (0.002, 90.0)	0.832

cOR: crude odds ratio
aOR: adjusted for age, prepregnancy BMI, employment status, and other THs

5.4 Conclusion

To conclude, we developed an HPLC-QqQ-MS method for TH analysis in placenta, which showed high sensitivity and selectivity. With minor modifications, the methods can be easily adopted for other tissue and blood samples. We did not observe significant associations between placental THs and GDM in a Chinese birth cohort. However, since the sample size in this study is low, the statistical power is limited. These results should be further confirmed with more placenta samples.

Chapter 6 Sample pooling strategy in T2D Exposome Wide Association Studies of POPs in serum

Abstract

Exposome Wide Association Studies (EWAS) require large sample numbers to identify environmental risk factors, which dramatically increases costs and research workload. Recent studies proposed a sample pooling strategy to reduce the complexity. In this chapter, we applied this approach for an EWAS study. Serum samples from 132 incident cases with type 2 diabetes mellitus (T2D) and 264 controls were collected from the CARLA study ("Cardiovascular Disease-Living and Aging in Halle", 2002-2006, East Germany). PCBs and OCPs were analyzed. Initially, the POPs were measured in individual samples and only PCB-138 and -153 were positively associated with T2D. We hypothesized that in pooled samples more chemicals might show a significant difference between cases and controls. The residues were pooled in cases and controls, respectively, and the POPs were determined in seven replicates. The volumes of the pooled residue in cases and controls were similar (Mann-Whitney U test, p=0.268). The mean concentrations of the POPs in the pooled samples were similar to those in the individual sample. In the pooled samples, OCPs and PCBs were normally distributed. PCB-105, -114, -118, -138, -153, -156, -157, -167, -180, -189, β-HCH, HCB, octachlorostyrene, 4,4'-DDT, 2,4'-DDT, 4,4'-DDE, 2,4'-DDE, oxychlordane, and cis-Heptachlor epoxide were significantly different between pooled cases and pooled controls. Our results are consistent with previous findings, which found positive associations between T2D and PCB-105, -118, -138, -153, -156, -157, -180, β-HCH, HCB, 4,4'-DDT, 2,4'-DDT, 4,4'-DDE, 2,4'-DDE, oxychlordane and heptachlor epoxide. Therefore, our hypothesis can be accepted. The reason might be the larger amount of pooled samples which leads to a improved sensitivity for POP analysis. Pooling also enforces the disclosure of differences between cases and controls by eliminating the biological variance and by converting a log-normal distribution into a normal distribution. Although certain limitations exist, such as the loss of individual information, it is nonetheless a useful explorative tool in EWAS.

Author contribution:

6.1 Introduction

Exposome Wide Association Studies (EWAS) require large sample numbers to identify environmental risk factors, which dramatically increases costs and research workload. Recent studies proposed a sample pooling strategy to reduce the complexity[163]. Here, we applied this approach for an EWAS study.

6.2 Materials and methods

6.2.1 Sample collection

Serum samples from 132 incident cases with type 2 diabetes mellitus (T2D) and 264 controls were collected from the CARLA study ("Cardiovascular Disease-Living and Aging in Halle", 2002-2006, East Germany). Details on study design have been described elsewhere [234, 235]. Previous study only observed significant associations of T2D with PCB-138 and -153. We hypothesized that in pooled samples more chemicals might show a significant difference between cases and controls.

The residual serum samples were pooled into a control and case sample. 1mL each of these pools were determined in seven replicates for organochlorinated pesticides (OCP) and polychlorinated biphenyls (PCB) employing isotope dilution methodology. The volumes of the pooled residue in cases and controls were similar (Mann-Whitney U test, p=0.268).

6.2.2 Chemicals

All solvents used were of trace analysis quality and purchased from LGC Standards GmbH (Wesel, Germany). This also applied for the anhydrous sodium sulfate, silica and alumina

adsorbents. The internal standard solutions of OCP ($^{13}C_6$-pentachlorobenzene, $^{13}C_6$-α-HCH, $^{13}C_6$-γ-HCH, $^{13}C_6$-β-HCH, $^{13}C_6$-delta-HCH, $^{13}C_6$-pentachloroanisole, $^{13}C_6$-hexachlorobenzene, $^{13}C_{10}$-heptachlor, $^{13}C_6$-octachlorostyrene, $^{13}C_{10}$-oxy-chlordane, $^{13}C_{10}$-heptachloroepoxide, $^{13}C_{12}$-o,p'-DDE, $^{13}C_{12}$-p,p'-DDE, $^{13}C_{12}$-trans-chlordane, $^{13}C_9$-endosulfan-I, $^{13}C_9$-endosulfan-II, D_8-p,p'-DDD, $^{13}C_{12}$-dieldrin, $^{13}C_{12}$-o,p'-DDT, $^{13}C_{12}$-p,p'-DDT, $^{13}C_{10}$-mirex), $^{13}C_{12}$-PCB (PCB-28, PCB-52, PCB-77, PCB-81, PCB-101, PCB-105, PCB-114, PCB-118, PCB-123, PCB-126, PCB-138, PCB-153, PCB-156, PCB-157, PCB-167, PCB-169, PCB-180, PCB-189), $^{13}C_{12}$-PCDD/F (2,3,7,8-TCDF, 2,3,7,8-TCDD, 2,3,4,7,8-PeCDF, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 2,3,4,6,7,8-HxCDF, 1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,4,6,7,8-HpCDF, 1,2,3,4,6,7,8-HpCDD, OCDF, OCDD) in nonane were added before extraction. For determination of the recoveries of the quantification standards, which might be lost during the extraction and clean-up, recovery standards for OCP (pentachlortoluol (PCT), $^{13}C_{12}$-1,2,3,7,8,9-HxCDD), PBDE ($^{13}C_{12}$-BDE-138), PCB ($^{13}C_{12}$-PCB-70, $^{13}C_{12}$-PCB-111, $^{13}C_{12}$-PCB-170), and PCDD/F ($^{13}C_{12}$-1,2,3,4-TCDD, $^{13}C_{12}$-1,2,3,7,8,9-HxCDD) were added in each vial of purified sample. The ^{13}C-labelled standards were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) or Wellington Laboratories (Guelph, Ontario, Canada).

6.2.3 Sample cleanup

The extraction was carried out on an Accelerated Solvent Extractor (ASE 200, Dionex GmbH, Idstein, Germany). 2 mL of human serum was well homogenized with Chem Tube-Hydromatrix (Agilent Technologies, USA) to fill up a 22 mL extraction cell and spiked with ^{13}C-labelled standard mixtures (OCP, PBDE, PCB and PCDD/F). For extraction toluene was used at extraction conditions of 120°C and pressure of 12 MPa. Two static cycles of 10 min were applied for a complete extraction. After the extraction step, the organic phase was passed over anhydrous sodium sulfate to remove water.

The volume of the extract was reduced to approx. 1 mL by the help of vacuum rotary and cleaned-up on an open double-layer glass column containing 5 g silica and 2.5 g alumina B (deactivated with 3% distilled water). The compounds were eluted with 50 mL n-hexane/dichloromethane (1:1). In order to avoid blanks and to eliminate impurities the packed

column was previously rinsed with 30 mL of *n*-hexane/dichloromethane (1:1). Afterwards, the sample was reduced to ca. 0.5 mL with a gentle stream of nitrogen and carefully transferred into the micro-insert of a GC vial and finally reduced to a volume of 20 µL.

6.2.4 Instrumental analysis

OCP, PCB, and PCDD/F analysis was performed by high resolution gas chromatography–high resolution mass spectrometry (HRGC/HRMS); the instrumental parameters are listed in Table 6.1. The MS was operated in SIM mode and the two most intense ions of the molecular ion cluster or an abundant fragment ion were monitored for the unlabeled and labelled isomers.

6.2.5 Statistical analysis

The concentrations of POPs measured in the pooled case and control replicates were compared using Welch t-test. $P < 0.05$ was considered as significant.

Table 6.1 GC/MS parameter for the isomer specific detection of OCP, PCB, PCDD/F.

	PCDD/Fs	PCBs	OCPs
GC			
Type	Agilent 6890	Agilent 5890 Series II	Agilent 5890 Series II
Column	Rtx-Dioxin2, 40 m, 0.18 mm ID, 0.18 µm film thickness (Restek)	Stx-CLPesticides2, 30 m, 0.25 mm ID, 0.2 µm film thickness (Restek)	Stx-CLPesticides2, 30 m, 0.25 mm ID, 0.2 µm film thickness (Restek)
Temperature program	130°C, 1.5 min, 25°C/min, 205°C, 4°C/min, 310°C, 15 min	90°C, 1.5 min, 20°C/min, 170°C, 7.5 min, 3.5°C/min, 285°C, 20°C/min, 320°C, 10 min	60°C, 1.5 min, 12°C/min, 140°C, 5°C/min, 300°C, 10 min
Carrier gas	H_2, constant flow: 1.2 mL/min	H_2, head pressure: 16 psi	H_2, head pressure: 16 psi
Injector	cooled injection system CIS 4 (Gerstel)	cooled injection system CIS 3 (Gerstel)	cooled injection system CIS 3 (Gerstel)
Temperature program	120°C, 12°C/s, 300°C, 5 min	120°C, 12°C/s, 300°C, 5 min	120°C, 12°C/s, 300°C, 5 min

| Temperature transfer line	300 °C	300 °C	300 °C
Injection volume	1 µL pulsed splitless	1 µL splitless	0.8 µL splitless
MS			
Type	MAT 95XL (Thermo)	MAT 95S (Thermo)	MAT 95S (Thermo)
Ionization mode	EI, 45 eV, 260°C	EI+, 47 eV, 260°C	EI+, 47 eV, 260°C
Resolution	> 9000	> 8000	> 8000

6.3 Results and discussion
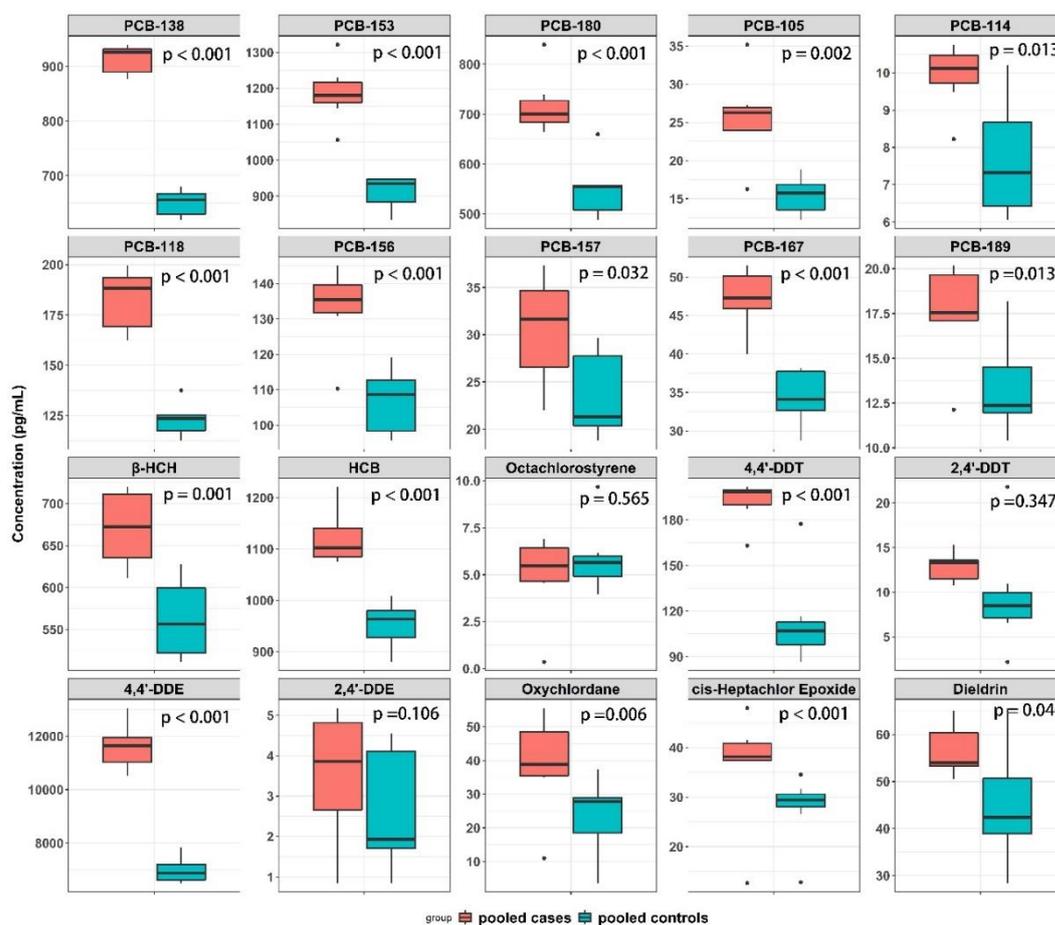
Fig. 6.1 Concentrations of POPs measured in pooled cases and pooled controls. N=7, Welch t-test. P-values of the comparison are indicated in the figure.

The mean concentrations of the POPs in the pooled samples were similar to those in the individual sample. In the pooled samples, OCPs and PCBs were normally distributed. As

shown in Fig. 6.1, PCB-105, -114, -118, -138, -153, -156, -157, -167, -180, -189, β-HCH, HCB, octachlorostyrene, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, oxychlordane, and cis-Heptachlor epoxide were significantly different between pooled cases and pooled controls.

Our results are consistent with previous findings, which found positive associations between T2D and PCB-105, -118, -138, -153, -156, -157, -180, β-HCH, HCB, p,p'-DDT, o,p'-DDT, p,p'-DDE, o,p'-DDE, oxychlordane and heptachlor epoxide [110, 113, 236, 237].

Our hypothesis can be accepted. The reason might be the larger number of pooled samples which leads to a higher sensitivity for POP analysis. Pooling also enforces the disclosure of differences between cases and controls by eliminating the biological variance and by converting a log-normal distribution into a normal distribution.

6.4 Conclusion

To conclude, we observed significant differences of certain POP levels in pooled serum from T2D and control participates. This study suggested that sample pooling strategy can be used as an explorative approach in epidemiological studies. Although certain limitations exist, such as the loss of individual information, it is nonetheless a useful explorative tool in EWAS.

Chapter 7 Placental distribution and transfer of thyroid hormones, elements, persistent organic pollutants and monoamines: a pilot study

Abstract

Placentome has been used to assess intrauterine environment. Reliability in the use of placental biomarkers requires an understanding of their distributions. A simple and proper placenta sampling scheme off delivery room is warranted. In this chapter, we developed a continuous cooling chain protocol off delivery room and cryo-subsampling method for placenta sampling. The placental distribution of THs, elements, POPs, monoamines, and vitamin E were assessed using such subsamples. In human placentome, T_4, Cd, Se, Zn, Cu, Fe, Ca, K, and Mg levels were higher in placenta than in umbilical cord, while Pb and Mn were concentrated in human cord. In porcine placenta, T_4, rT_3, 3,3'-T_2, Cd, Pb, Zn, K, and Al levels were higher in the cord. The intraclass correlation coefficient (ICC) was < 0.4 for 3,3',5-triiodo-L-thyronine, rT_3, and 7 elements in human basal plate, indicating low reliability. rT_3, Cd, Zn, Mn, and Cu were significantly concentrated in the central region in human placenta, while higher levels of As, Cd, Cr, and Al were found in the periphery region in porcine placenta. The high coefficient of variation (CV%) of certain THs in tissues were consistent with their low ICCs. PCBs and PBDEs showed good reliability (ICC: 0.40–0.98) except PCB-81, -126, and BDE-208, while PCDD/Fs showed poor reliability (ICC: 0.07–0.31). These results highlight the complexity of perinatal environment and placenta sampling. This study provides a novel and simple sampling approach in investigating placental exposomics.

7.1 Introduction

In this study, we conducted a comprehensive assessment of the regional distribution of various constituents in the maternal portion of placenta and umbilical cord. These chemicals include hormones (T_4, T_3, rT_3, and 3,3'-T_2), POPs (PBDEs, PCBs, PCDD/Fs, and OCPs), and metals (Pb, Hg, As, Cd, Cr, Mn, Zn, Se, Cu, Fe, Ca, K, Mg, Na and Al). Placenta and umbilical cord tissues from both humans and pigs were adopted to validify the results. The regional distribution was evaluated using the variations of chemical levels in placenta, as well as the comparations between different regions (including peri-insertional, mid-disc and periphery regions). The variations of THs were compared to those in repeated measurements of a reference placenta material. Finally, the TH and metal levels in placenta and umbilical cord samples were compared to assess their transplacental passage.

7.2 Materials and methods

7.2.1 Sample collection

Five human placentas with umbilical cords and cord blood were collected from the Women's Hospital of Tübingen University, Germany. The healthy women showed similar demographic characteristics. Three porcine placentas with umbilical cords were obtained from the Technical University of Munich, Germany. The type of chemical transfer in human and porcine placenta might be different because human placenta is hemochorial while porcine placenta is epitheliochorial. We included placentomes with different structures to compare the

results. Appropriate precautions were taken to avoid contamination. Placenta and umbilical cord were separated immediately and frozen at < -20°C in a container, transported to the Helmholtz Center Munich, Germany, and stored at < -80°C until further processing.

A drilling machine was used to collect finely powdered spot samples across the basal plate. As shown in Fig. 7.1, at the temperature of < -150°C in liquid nitrogen, 11–15 and 3–6 spot samples were collected from the basal plate of each placenta and umbilical cord, respectively.
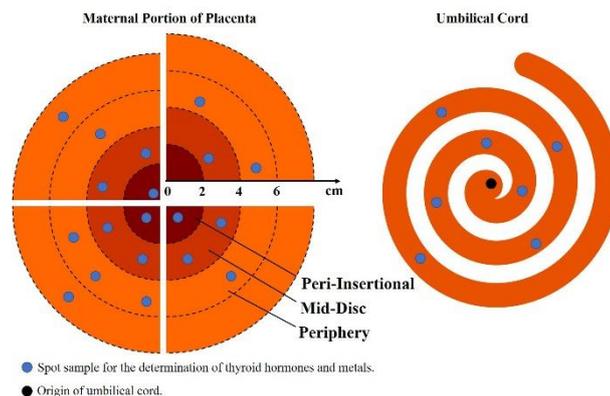

Fig. 7.1 Schematic diagram shows the spot sampling in placenta and umbilical cord.

The number of spot samples was determined by the size of the tissues, which were 67, 17, 30, and 17 in human placenta, human umbilical cord, porcine placenta, and porcine umbilical cord, respectively (Table 7.1).

Table 7.1 Number of spot samples in each human and porcine tissue.

tissue		Placenta basal plate	Umbilical cord
Human	No. 1	13	3
	No. 2	13	3
	No. 3	15	3
	No. 4	13	3
	No. 5	13	5
Pig	No. 1	11	5
	No. 2	10	6
	No. 3	9	6

The wet weight of each spot sample was < 2 g. Seven THs, 15 elements, and 9 monoamines were measured in these spot samples. At least 10 g sample was needed for POP analysis, thus the residual placenta was cut into 4 parts (Fig. 7.1), each part was homogenized, and seventy-four POPs were measured. About 10 mL of cord blood was collected from each umbilical cord, in which THs and monoamines were determined.

This study followed the Helsinki II declaration (World Medical Association 2004) and was approved by the local ethic boards. Animal experiments were approved by the government of Upper Bavaria and handled following the German Animal Welfare Act.

7.2.2 Chemical analysis

(1) Thyroid hormones

T_4, T_3, rT_3, 3,3'-T_2, T_1, and 3-T_1AM, $^{13}C_6$-T_3 and $^{13}C_6$-rT_3 were from Sigma-Aldrich (St. Louis, MO, USA). 3,5-T_2 and $^{13}C_6$-3,5-T_2 were from Santa Cruz Biotechnology (Dallas, Texas, USA). $^{13}C_6$-T_4 and $^{13}C_{12}$-T_4 were purchased from Cambridge Isotope Laboratories (Andover, MA). Bond Elut Plexa PCX cartridges (60 mg, 3 mL) were from Agilent Technologies (Santa Clara, CA, USA). Finally, a protection solution consisted of 10 mg/mL citric acid monohydrate, L-(+)-ascorbic acid and R, R-dithiothreitol was prepared daily in water.

A 100-mg spot sample was cleaned up with solid/liquid extraction, liquid/liquid extraction, and weak cation exchangers solid phase extraction (SPE) [37]. The quantification was achieved by liquid chromatography mass spectrometry (LC-MS). A protection solution (including citric acid monohydrate, L-(+)-ascorbic acid and R-dithiothreitol) was added to inhibit TH degradation. No degradation of THs was observed during the analytical process. The method quantification limits (MQL) of T_4, T_3, rT_3, and 3,3'-T_2 were 0.7, 0.3, 0.2, and 0.04 ng/g, respectively. The spike-recoveries for the compounds were 81.0–105%, with a coefficient of variation (CV) of 0.5–6.2%. The intra-day CVs and inter-day CVs were 0.5–6.80% and 1.19–6.38%, respectively.

For quality control, solvent blanks and standard solutions were regularly injected. The identification of target analytes was based on the retention times compared with internal standards and *m/z* ratios. A pooled human placenta spiked with 30 ng/g of T_3, rT_3 and T_2 was

employed as a reference material, which was measured repeatedly, and the results were considered as acceptable if the variance between different measurements was < 20%.

(2) Elements

Certified stock standard solutions for Cd, Hg, Pb and Se (1 mg/mL for each) were from CPI (Santa Rosa, CA, USA). A customized certified multi-element standard solution containing Cr, Zn, Mn, Cu, and Mg was purchased from Horiba Jobin Yvon (Pullach, Germany). HNO_3 was from Merck (Darmstadt, Germany). Liquid argon and oxygen (99.999%) were from Air Liquide (Gröbenzell, Germany). Deionized water (18.2 MΩ) used for the preparation of standards and samples were obtained from a Milli-Q Millipore filter system (Bedford, MA, USA).

A wide variety of elements, including As, Cd, Cr, Hg, Pb, Se, Zn, Mn, Cu, Fe, Ca, K, Mg, Na, and Al were targeted for analysis in this study. The method for sample cleanup and quantification were based on previous methodology with minor modifications [238, 239]. Briefly, a 100-mg sample was thawed slowly at 4°C and diluted with Milli-Q water, which contains ^{103}Rh as internal standard. The diluted samples were used for elemental analysis. The quantification of elements was achieved on an inductively coupled plasma optical emission spectrometry (ICP-OES) 'Spectro Ciros Vision' system (SPECTRO Analytical Instruments GmbH & Co. KG, Kleve, Germany) and an ELEMENT 2, Thermo-Electron (Bremen, Germany) ICP-sf-MS instrument.

(3) Persistent organic pollutants (POPs)

The quantification standard solutions of ^{13}C$_{12}$-PCB, ^{13}C$_{12}$-PCDD/F, ^{13}C$_{12}$-PBDE, recovery standard solutions of ^{13}C$_{12}$-1,2,3,7,8,9-HxCDD, ^{13}C$_{12}$-BDE-138, ^{13}C$_{12}$-PCB-70, ^{13}C$_{12}$-PCB-111, ^{13}C$_{12}$-PCB-170, ^{13}C$_{12}$-1,2,3,4-TCDD, and ^{13}C$_{12}$-1,2,3,7,8,9-HxCDD were purchased from Cambridge Isotope Laboratories (Andover, MA, USA) or Wellington Laboratories (Guelph, Ontario, Canada).

About 10 g placenta sample was well homogenized with Chem Tube-Hydromatrix (Agilent Technologies, USA) to fill up a 33 mL extraction cell and spiked with ^{13}C-labelled standard mixtures (PBDE, PCB and PCDD/F). For extraction by pressurized liquid extraction (Dionex ASE 200, USA) n-hexane/acetone (3:1, v/v) was used at conditions of 120°C and pressure of

12 MPa. Two static cycles of 10 min were applied for a complete extraction. After the extraction step, the organic phase was passed over anhydrous sodium sulfate to remove water. The volume of the extract was reduced to approx. 5 mL by the help of vacuum rotary and cleaned-up by use of an automated system (DEXTech, LCTech, Germany), where the sample was passed and fractionated over an acidic silica, alumina and carbon column. Afterwards, the two resulting fractions (fraction 1: PCBs, PBDEs; fraction 2: non-ortho PCBs, PCDD/Fs) were reduced to ca. 0.5 mL with a gentle stream of nitrogen and carefully transferred into the micro-insert of a GC vial, spiked with the recovery standard solutions and finally reduced to a volume of 20 µL. The analyses of PBDEs, PCBs, and PCDD/Fs were performed by high resolution gas chromatography–high resolution mass spectrometry (HRGC/HRMS); the instrumental parameters are listed in Table 7.2. The MS was operated in SIM mode and the two most intense ions of the molecular ion cluster or an abundant fragment ion were monitored for the unlabeled and labelled isomers.

(4) Monoamines

The chemicals, instrumentation and chromatographic conditions were reported before [240].

A 200-mg portion of placenta sample was weighted and placed into a 15-mL polypropylene centrifuge tube. Two hundred micro liter of $HClO_4$ (0.3 M) and 4 µL of 3,4-dihydroxybenzylamine (internal standard) were added, followed by homogenization using ultrasonication on ice for 30 sec. The homogenate was centrifuged (7899 g, 4 °C, 10 min), and the supernatant was transferred into a sample vial and measured with HPLC-EcD.

For blood sample clean-up, the protocol from RECIPE for the extraction of catecholamines from human plasma was used (https://www.recipe.de/en/products_hplc_diagn_01000.html). The Recipe ClinRep® complete kit contains all the necessary chemicals and materials for such extraction. 20 µL of the extracted catecholamines was injected into the HPLC-EcD system.

7.2.3 Data analysis

The distribution variability of chemicals in placentome tissues was assessed by intraclass correlation coefficient (ICC), which is defined as the percent of total variance explained by between-tissue variance. We used a one-way random-effects ANOVA model to calculate the

ICC for each chemical independently. Values of the ICC < 0.40; 0.40–0.75; and ≥ 0.75 suggest poor, fair to good, and excellent reliability, respectively [241, 242]. To improve statistical robustness, only POP congeners that have a detection frequency (DF) of $\geq 60\%$ were analyzed. Concentrations below the limit of detection (LOD) were substituted with LOD divided by square root of 2. All analyses were conducted on log10-transformed values due to their non-normal distribution. Additionally, a pooled placenta sample spiked with 30 ng/g of T_3, rT_3, 3,3'-T_2, 3,5-T_2, T_1, and 3-T_1AM was measured repeatedly to estimate the measurement variation of THs. The TH variances in spot samples were compared with the measurement variances using Mann-Whitney U test.

Table 7.2 GC/MS parameters for the isomer specific detection of POPs.

	PCDD/Fs	PCBs	PBDEs
GC Type	Agilent 6890	Agilent 6890	Agilent 6890
Column	Rtx-Dioxin2, 40 m, 0.18 mm ID, 0.18 μm film thickness (Restek)	Rtx-Dioxin2, 40 m, 0.18 mm ID, 0.18 μm film thickness (Restek)	Rtx-1614, 15 m, 0.25 mm ID, 0.1 μm film thickness (Restek)
Temperature program	130°C, 1.5 min, 25°C/min, 205°C, 2°C/min, 245°C, 5°C/min, 310°C, 10 min	90°C, 1.5 min, 15°C/min, 200°C, 4°C/min, 300°C, 10 min	75°C, 1.5 min, 18°C/min, 210°C, 8°C/min, 310°C, 5 min
Carrier gas	helium	helium	helium
Flow (mL/min)	1.2	1.2	1.6
Injector	cooled injection system CIS 4 (Gerstel)	cooled injection system CIS 4 (Gerstel)	cooled injection system CIS 4 (Gerstel)
Temperature transfer line	310°C	300°C	320°C
Injection volume	1 μL pulsed splitless	1 μL splitless	1 μL splitless
MS Type	MAT 95XL (Thermo)	MAT 95XL (Thermo)	MAT 95XL (Thermo)

Ionization mode	EI+, 45 eV, 260°C	EI+, 47 eV, 260°C	EI+, 47 eV, 260°C
Resolution	> 9000	> 9000	> 8000

According to their locations on placenta, the spot samples were divided into three groups: peri-insertion (< 2 cm), mid-disc (2–4 cm), and peripheral region (> 4 cm) (Fig. 7.1). TH and element levels in these regions were compared using Kruskal-Wallis test.

The trans-placental transfer of THs and elements were evaluated by comparing their concentrations in basal plate and umbilical cord tissues using Mann-Whitney U test. The concentration ratio between paired umbilical cord and placenta (C_c/C_p) was calculated to estimate the likelihood of bioaccumulation. Monoamines were measured in placenta, umbilical cord and cord blood to investigate their origin and trans-placental properties.

All statistical analyses in this study were executed using R (version 3.4.2; R Foundation for Statistical Computing, Vienna, Austria). A p-value < 0.05 was considered as significant.

7.3 Results

Table 7.3, Table 7.4 and Fig 4.2 present the results of TH and elemental analyses in human and porcine placentome, respectively.

The DFs of T_4, T_3, and rT_3 in human placentome were 100% except T_3 in umbilical cords (27.8%). The mean levels were 42.3 ng/g fresh weight (fw) T_4, 0.66 ng/g fw T_3, and 2.74 ng/g fw rT_3, and < LOD for other THs in human basal plate, and 19.1 ng/g fw T_4, 0.52 ng/g fw T_3, 2.41 ng/g fw rT_3, and < LOD for other THs in human umbilical cord. The DFs of T_4, T_3, rT_3, and 3,3'-T_2 in porcine placentome were > 96%. The mean levels were 11.3 ng/g fw T_4, 0.70 ng/g fw T_3, 1.23 ng/g fw rT_3, 2.34 ng/g fw 3,3'-T_2, and < LOD for other THs in porcine basal plate, and 25.5 ng/g fw T_4, 0.63 ng/g fw T_3, 2.08 ng/g fw rT_3 and 3.55 ng/g fw 3,3'-T_2 in porcine umbilical cord. T_4, T_3, and rT_3 were quantified in human cord blood with concentrations of 28.7 ± 7.4, 0.53 ± 0.26, 0.91 ± 0.18 ng/mL (mean ± SD), respectively.

The DFs of elements were > 96% in all spot samples. The concentrations of elements in human basal plate, human cord, porcine basal plate, and porcine cord were 3.27 ng/g fw (As)–

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1.99 mg/g fw (K), 1.40 ng/g fw (Cd)–1.85 mg/g fw (Na), 0.98 ng/g fw (Cd)–1.95 mg/g fw (Na), and 3.48 ng/g fw (As)–1.74 mg/g fw (Na), respectively (Table 7.3 & 4.4).
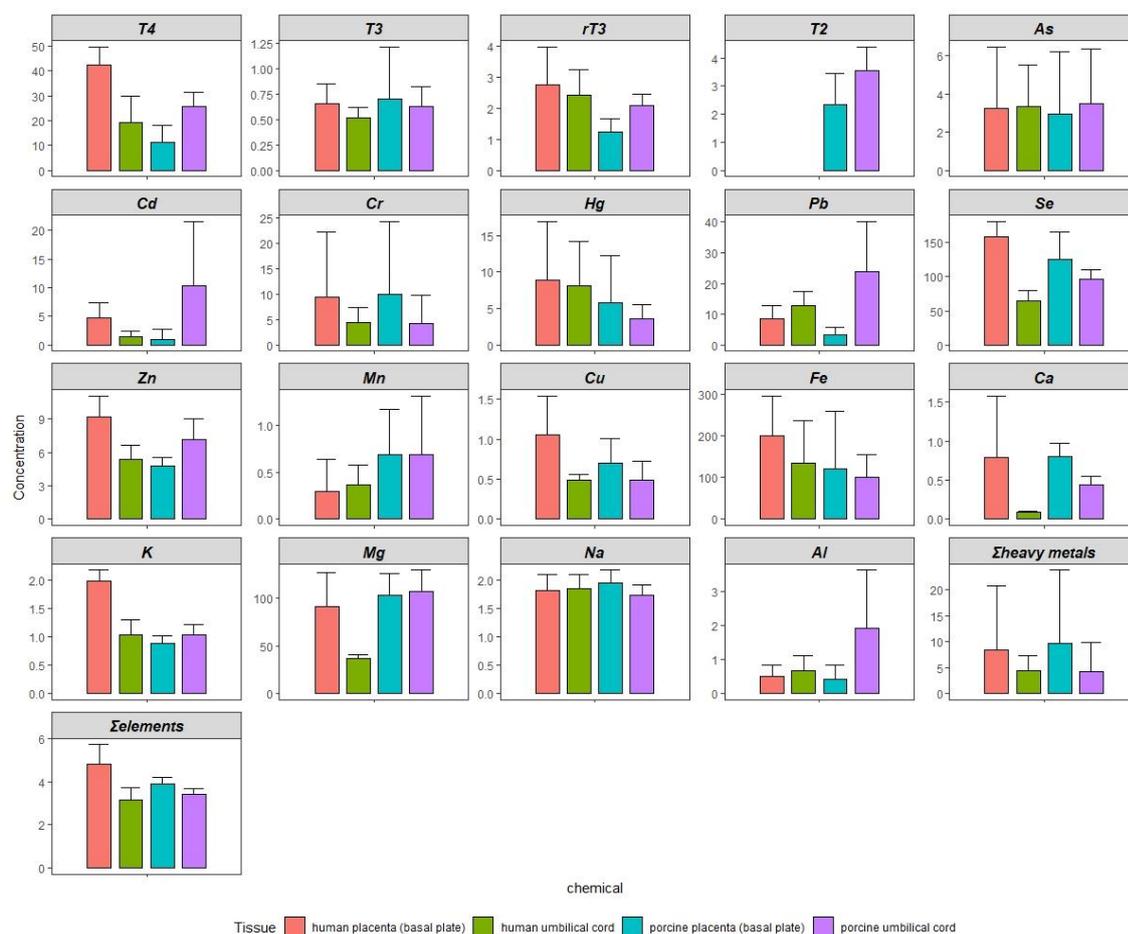

Fig. 7.2. Description of the THs and metals measured in human basal plate, umbilical cord, porcine basal plate, and porcine umbilical cord. The units are ng/g fw for T_4, T_3, rT_3, 3,3'-T_2, As, Cd, Hg, Pb, Se, μg/g fw for Cr, Zn, Mn, Cu, Fe, Mg, Al and sum heavy metals, mg/g fw for Ca, K, Na, and sum elements. 3,3'-T_2 was only quantified in porcine basal plate and porcine umbilical cord.

In total, 74 POPs including 17 polychlorinated dibenzo-*p*-dioxins and furans (PCDD/Fs), 18 polychlorinated biphenyls (PCBs), and 39 brominated flame retardants (BFRs) were determined in human placenta, in which 5 PCDD/Fs, 14 PCBs, and 4 BFRs had a DF of > 60%. The mean concentrations were 0.03–0.10, 0.07–78.4, and 0.96–4.52 pg/g fw for PCDD/Fs, PCBs, and BFRs, respectively. Besides, the World Health Organization toxic equivalents (WHO-TEQ) of PCDD/Fs and PCBs were 0.04–0.06 and 0.03 pg/g fw, respectively.

Table 7.3 Descriptive statistics and intraclass correlation coefficients (ICCs) of THs and elements in human placentome.

	Human basal plate			Human umbilical cord			C_c/C_p	p^b
	Mean (SD)	Median (Range)	ICC[a]	Mean (SD)	Median (Range)	ICC[a]	(mean (SD))	
T_4 (ng/g)	42.3 (7.2)	42.4 (24.3–57.6)	0.52	19.1 (11.2)	15.9 (5.79–47.2)	0.06	0.46 (0.11)	< 0.001
T_3 (ng/g)[c]	0.66 (0.20)	0.65 (0.34–1.31)	0.06	0.52 (0.11)	0.49 (0.40–0.68)	—	—	—
rT_3 (ng/g)	2.74 (1.23)	2.97 (0.48–4.86)	0.26	2.41 (0.85)	2.42 (1.16–4.45)	0.31	0.88 (0.41)	0.151
As (ng/g)	3.27 (3.21)	2.03 (0.55–15.4)	0.37	3.34 (2.27)	2.48 (1.51–11.0)	0.21	1.45 (1.14)	0.142
Cd (ng/g)	4.66 (2.70)	4.16 (1.01–15.1)	0.04	1.40 (0.97)	1.04 (0.46–4.14)	0.39	0.46 (0.37)	< 0.001
Cr (µg/g)	9.46 (12.7)	3.86 (0.08–60.9)	0.36	4.46 (2.97)	3.70 (1.06–12.5)	0.07	0.72 (0.41)	0.854
Hg (ng/g)	8.86 (8.13)	6.42 (0.38–37.2)	0.68	8.08 (6.32)	5.30 (2.14–22.6)	0.62	1.51 (0.72)	0.699
Pb (ng/g)	8.57 (4.39)	7.47 (2.36–22.8)	0.10	12.9 (4.67)	13.5 (6.03–21.6)	0.15	6.80 (10.0)	< 0.001
Se (ng/g)	159 (22)	163 (96.1–201)	0.42	65.3 (15.7)	61.1 (43.9–96.0)	0.12	0.43 (0.06)	< 0.001
Zn (µg/g)	9.21 (1.91)	8.84 (6.57–16.5)	0.38	5.35 (1.39)	4.82 (4.27–9.76)	0.40	0.62 (0.17)	< 0.001
Mn (µg/g)	0.30 (0.34)	0.15 (0.59–1.67)	0.37	0.37 (0.22)	0.29 (0.11–0.87)	0.60	1.75 (0.80)	0.01
Cu (µg/g)	1.06 (0.49)	0.93 (0.68–3.01)	0.29	0.49 (0.07)	0.48 (0.37–0.64)	0.04	0.50 (0.09)	< 0.001
Fe (µg/g)	200 (97)	180 (67.8–517)	0.46	135 (105)	101 (31.2–371)	0.35	0.62 (0.29)	< 0.01
Ca (mg/g)	0.79 (0.80)	0.51 (0.13–4.02)	0.61	0.09 (0.01)	0.09 (0.08–0.10)	0.41	0.15 (0.08)	< 0.001
K (mg/g)	1.99 (0.20)	1.97 (1.64–2.38)	0.47	1.04 (0.28)	0.99 (0.63–1.55)	0.19	0.52 (0.04)	< 0.001

Mg (µg/g)	91.2 (36.0)	80.5 (60.5–253)	0.59	37.0 (4.0)	36.8 (29.6–46.9)	0.18	0.44 (0.10)	< 0.001
Na (mg/g)	1.83 (0.27)	1.82 (0.21–2.23)	0.76	1.85 (0.25)	1.86 (1.39–2.25)	0.28	1.02 (0.16)	0.763
Al (µg/g)	0.50 (0.34)	0.39 (0.09–1.68)	0.43	0.67 (0.44)	0.55 (0.17–1.94)	0.15	1.99 (1.13)	0.090
Σ heavy metals (µg/g)[d]	8.50 (12.4)	3.49 (0.02–60.9)	0.04	4.48 (2.97)	3.72 (1.07–12.5)	0.61	0.85 (0.49)	0.776
Σ elements (mg/g)[e]	4.84 (0.90)	4.66 (3.43–8.34)	0.31	3.17 (0.56)	2.99 (2.34–4.23)	0.11	0.65 (0.10)	< 0.001

Abbreviations: ICC, intraclass correlation coefficient. C_c, concentration in human umbilical cord. C_p, concentration in human placenta. IQR, interquartile range showing the 25th and 75th percentile values. [a]Variances estimated for each chemical using a one-way random-effects ANOVA model; calculations were performed on log10-transformed data; ICC= between-tissue variance/total variance. [b]Difference between human placenta and umbilical cord analyzed by Mann-Whitney U test. [c]The ICC value of T_3 in human umbilical cord was not calculated due to the low detection frequency. [d]The sum of As, Cd, Cr, Hg, and Pb. [e]The sum of all the elements measured.

As shown in Table 7.3, the levels of rT_3, As, Cr, Hg, Na, Al, and Σ heavy metals were similar between human basal plate and umbilical cord. T_4 ($p < 0.001$), Cd ($p < 0.001$), Se ($p < 0.001$), Zn ($p < 0.001$), Cu ($p < 0.001$), Fe ($p < 0.01$), Ca ($p < 0.001$), K ($p < 0.001$), Mg ($p < 0.001$), and Σ elements ($p < 0.001$) levels were significantly higher in human basal plate than in umbilical cord, while Pb ($p < 0.01$) and Mn ($p = 0.01$) showed higher level in umbilical cord with a C_c/C_p value of 6.80 and 1.75, respectively.

In porcine tissues, T_3, As, Cr, Hg, Mn, Fe, Mg, and Σ heavy elements showed similar concentrations between basal plate and umbilical cord. Se ($p = 0.01$), Cu ($p < 0.01$), Ca ($p < 0.001$), and Na ($p < 0.01$) showed higher levels in basal plate. T_4 ($p < 0.001$), rT_3 ($p < 0.001$), 3,3'-T_2 ($p < 0.001$), Cd ($p < 0.001$), Pb ($p < 0.001$), Zn ($p < 0.001$), K ($p < 0.01$), and Al ($p < 0.001$) showed higher levels in umbilical cord. The C_c/C_p values of these chemicals were 1.16–14.2 (Table 7.4).

As shown in Tables 7.3 & 7.4, in human basal plate, the ICC measure of reliability was good (0.42–0.76) for T_4 and 8 elements (Hg, Se, Fe, Ca, K, Mg, Na, and Al). ICC was poor (0.04–0.38) for T_3, rT_3 and remaining 7 elements (As, Cd, Cr, Pb, Zn, Mn, and Cu). In human umbilical cord, reliability was moderate (0.40–0.62) for 4 elements (Hg, Zn, Mn, and Ca) and Σ heavy metals, while other chemicals showed low reliability (0.04–0.35). In porcine basal plate, the reliability was good (0.48–0.73) for T_4 and 2 elements (Mg, and Na). In porcine umbilical cord, ICCs was good (0.42–0.66) for T_4, rT_3, and 5 elements (As, Cr, Se, Mn, and Cu). Fig. 7.3 compares the biological and measurement variations of THs, the variances of T_3 ($p < 0.01$) and rT_3 ($p < 0.01$) in human basal plate, and T_4 ($p < 0.01$) in human umbilical cord were significantly higher than the measurement variances. The variances of T_4 ($p < 0.05$), T_3 ($p < 0.05$), rT_3 ($p < 0.05$), and 3,3'-T_2 ($p < 0.05$) in porcine basal plate, as well as T_3 ($p < 0.05$) and 3,3'-T_2 ($p < 0.05$) in porcine umbilical cord were higher than the measurement variances.

Norepinephrine (NE) and dopamine (DA) were quantified in cord blood with concentrations of 0.21 ± 0.10 and 1.78 ± 0.74 ng/mL (mean $\pm$ SD), respectively. Other monoamines were < LOD in all samples of basal plate, umbilical cord and cord blood.

Table 7.4 Descriptive statistics and intraclass correlation coefficients (ICCs) of THs and elements in porcine placentome.

	Porcine basal plate			Porcine umbilical cord			C_c/C_p	p^b
	Mean (SD)	Median (Range)	ICC[a]	Mean (SD)	Median (Range)	ICC[a]	(mean SD))	
T_4 (ng/g)	11.3 (6.8)	9.71 (4.20–36.9)	0.50	25.5 (5.9)	24.5 (17.4–35.4)	0.56	2.85 (1.46)	< 0.001
T_3 (ng/g)	0.70 (0.52)	0.59 (0.19–2.50)	0.36	0.63 (0.21)	0.55 (0.37–1.07)	0.19	1.18 (0.60)	0.722
rT_3 (ng/g)	1.23 (0.43)	1.16 (0.64–3.08)	0.21	2.08 (0.37)	2.02 (1.58–2.64)	0.66	1.84 (0.30)	< 0.001
3,3'-T_2 (ng/g)	2.34 (1.13)	2.06 (0.76–4.76)	0.23	3.55 (0.86)	3.48 (2.00–5.07)	0.24	1.70 (0.45)	< 0.001
As (ng/g)	2.95 (3.30)	1.67 (0.34–14.3)	0.10	3.48 (2.98)	2.08 (0.84–11.2)	0.42	1.38 (0.62)	0.211
Cd (ng/g)	0.98 (1.72)	0.63 (0.08–9.56)	0.24	10.3 (11.6)	4.85 (0.47–39.5)	0.14	14.2 (8.4)	< 0.001
Cr (µg/g)	10.0 (14.5)	3.69 (0.28–62.7)	0.05	4.20 (5.80)	1.62 (0.50–2.22)	0.56	0.55 (0.40)	0.189
Hg (ng/g)	5.81 (6.55)	4.35 (0.32–33.8)	0.06	3.54 (2.06)	3.29 (0.57–8.43)	0.11	0.87 (0.29)	0.145
Pb (ng/g)	3.40 (2.27)	2.98 (0.98–12.6)	0.03	23.9 (16.9)	19.0 (5.22–72.3)	0.02	7.69 (4.22)	< 0.001
Se (ng/g)	125 (41)	112 (82.4–252)	0.37	96.9 (13.4)	96.8 (74.5–125)	0.61	0.84 (0.13)	0.01
Zn (µg/g)	4.76 (0.82)	4.90 (3.07–6.13)	0.32	7.17 (1.90)	7.11 (4.94–12.6)	0.04	1.52 (0.05)	< 0.001
Mn (µg/g)	0.69 (0.49)	0.51 (0.27–2.30)	0.03	0.69 (0.65)	0.46 (0.20–2.21)	0.60	1.41 (1.16)	0.38
Cu (µg/g)	0.70 (0.31)	0.74 (0.11–1.30)	0.30	0.49 (0.23)	0.53 (0.12–0.91)	0.52	0.63 (0.21)	< 0.01
Fe (µg/g)	122 (139)	71.5 (20.8–659)	0.01	100 (56)	108 (18.7–242)	0.04	1.07 (0.48)	0.854
Ca (mg/g)	0.81 (0.17)	0.79 (0.51–1.21)	0.13	0.44 (0.12)	0.47 (0.15–0.59)	0.02	0.57 (0.11)	< 0.001

K (mg/g)	0.89 (0.13)	0.89 (0.65–1.12)	0.02	1.04 (0.18)	1.04 (0.78–1.47)	0.33	1.16 (0.17)	< 0.01
Mg (µg/g)	0.10 (0.02)	0.09 (0.07–0.15)	0.48	0.11 (0.02)	0.11 (0.06–0.14)	0.10	1.11 (0.14)	0.393
Na (mg/g)	1.95 (0.23)	2.03 (1.51–2.43)	0.73	1.74 (1.86)	1.71 (1.32–1.99)	0.34	0.91 (0.05)	< 0.01
Al (µg/g)	0.43 (0.42)	0.29 (0.08–2.30)	0.07	1.92 (1.74)	1.78 (0.04–7.25)	0.07	5.05 (2.33)	< 0.001
Σ heavy metals (µg/g)[c]	9.69 (14.3)	3.64 (0.009–62.7)	0.07	4.24 (5.81)	1.65 (0.53–22.2)	0.57	0.57 (0.39)	0.179
Σ elements (mg/g)[d]	3.88 (0.32)	3.85 (3.43–4.74)	0.30	3.44 (0.25)	3.39 (3.07–3.90)	0.42	0.85 (0.09)	< 0.001

Abbreviations: C_c, concentration in porcine umbilical cord. C_p, concentration in porcine placenta. IQR, interquartile range showing the 25th and 75th percentile values. [a]Variances estimated for each chemical using a one-way random-effects ANOVA model; calculations were performed on log10-transformed data; ICC= between-tissue variance/total variance. [b]Difference between porcine placenta and porcine umbilical cord analyzed by Mann-Whitney U test. [c]The sum of As, Cd, Cr, Hg, and Pb. [d]The sum of all the elements measured.
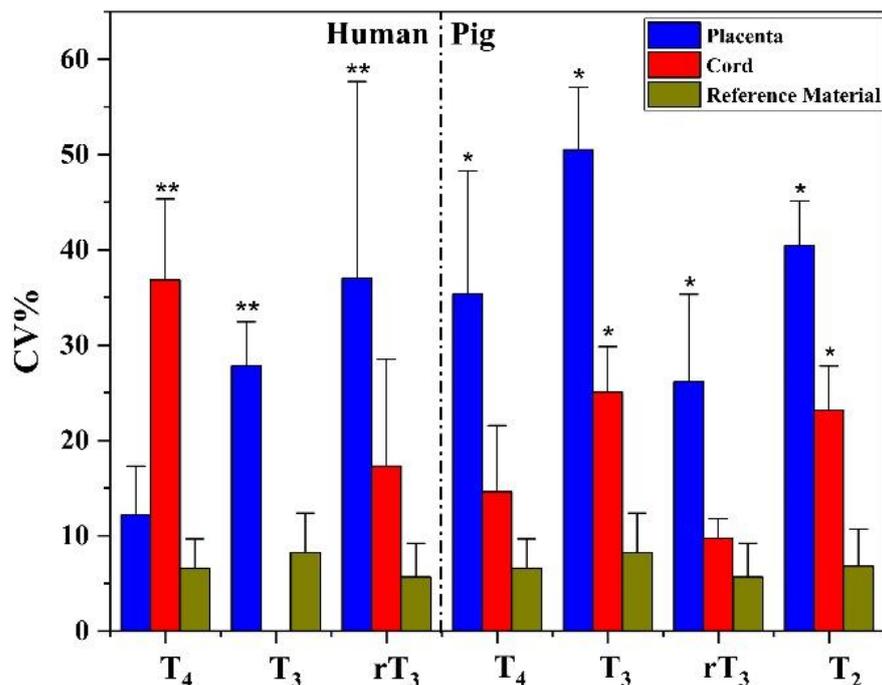
Fig. 7.3 Comparison of biological variation against measurement variation in human basal plate, human umbilical cord, porcine basal plate, and porcine umbilical cord. Variation was estimated by coefficient of variation (CV%). A pooled placenta spiked with 30 ng/g of T_3, rT_3, $3,3'-T_2$, $3,5-T_2$, T_1, and $3-T_1AM$ was used as reference material and measured repeatedly to estimate the measurement variances. The detection frequency of T_3 in human cord was $< 30\%$, and therefore not included in this analysis. $*p < 0.05$. $**p < 0.01$

As shown in Tables 7.5 and 7.6, the ICCs were low for all PCDD/Fs (0.08–0.31). PCBs showed good to excellent reliability (0.43–0.98) except PCB-81 and 126 (0.06–0.20). ICCs were good to excellent (0.40–0.94) for most BFR congeners while was poor (0.34) for BDE-208.

As shown in Figures 7.4 & 7.5, the ICCs of POPs were linearly related with their concentrations, while the ICCs of THs and elements showed poor linearity with their concentrations. This indicate that the poor reliability of certain POPs such as PCDD/Fs were the results of the low concentrations and high measurement variation. The low reliability of certain THs and elements, however, might be due to biological variances.

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Table 7.5 Descriptive statistics and measures of reliability of POPs with detection frequencies ≥ 60% in human placenta.

POPs	Detection frequency	LOD (pg/g fw)	Mean (SD) (pg/g fw)	Median (range) (pg/g fw)	ICC
PCDD/Fs					
1,2,3,4,6,7,8-HpCDD	80	0.001–0.004	0.09 (0.05)	0.08 (0.04–0.24)	0.07
2,3,4,7,8-PeCDF	90	0.001–0.002	0.10 (0.04)	0.08 (0.03–0.20)	0.16
1,2,3,4,7,8-HxCDF	65	0.001–0.004	0.05 (0.01)	0.05 (0.03–0.07)	0.17
1,2,3,6,7,8-HxCDF	65	0.001–0.004	0.03 (0.01)	0.02 (0.01–0.04)	0.31
1,2,3,4,6,7,8-HpCDF	65	0.001–0.002	0.05 (0.04)	0.03 (0.02–0.15)	0.08
Σ PCDD/Fs	100	—	0.32 (0.22)	0.29 (0.00–0.84)	0.16
PCDD/F_TEQ_1998	100	—	0.06 (0.04)	0.05 (0–0.20)	0.13
PCDD/F_TEQ_2005	100	—	0.04 (0.04)	0.03 (0–0.17)	0.15
PCBs					
PCB-28	100	≤ 0.1	8.62 (2.66)	8.91 (4.14–15.0)	0.58
PCB-52	60	4.8–6.6	7.99 (2.09)	7.79 (4.91–13.5)	0.58
PCB-81	60	0.004–0.01	0.07 (0.02)	0.07 (0.05–0.12)	0.06
PCB-105	100	≤ 0.1	5.30 (3.30)	4.15 (1.73–14.9)	0.75
PCB-114	100	≤ 0.1	0.52 (0.25)	0.49 (0.19–0.96)	0.65
PCB-118	100	≤ 0.1	13.0 (4.70)	12.8 (5.34–23.9)	0.72
PCB-123	95	≤ 0.35	0.88 (0.40)	0.84 (0.30–1.77)	0.46
PCB-126	100	≤ 0.1	0.25 (0.09)	0.25 (0.14–0.58)	0.20
PCB-138	100	≤ 0.1	78.4 (29.6)	87.2 (25.5–123)	0.91
PCB-153	100	≤ 0.1	47.4 (18.0)	51.7 (16.1–72.4)	0.92
PCB-156	100	≤ 0.1	5.08 (2.99)	4.42 (1.26–11.7)	0.93
PCB-157	100	≤ 0.1	0.83 (0.49)	0.70 (0.25–2.02)	0.76
PCB-167	100	≤ 0.1	1.99 (0.48)	2.04 (1.02–3.01)	0.70
PCB-180	100	≤ 0.1	42.5 (43.8)	23.1 (9.66–140)	0.98
Σ PCBs	100	—	212 (93)	211 (71.8–391)	0.92

PCB_TEQ_1998	100	—	0.03 (0.01)	0.03 (0.02–0.07)	0.44
PCB_TEQ_2005	100	—	0.03 (0.01)	0.03 (0.02–0.07)	0.43
BFRs					
BDE-153	75	0.6–0.7	3.71 (1.86)	4.24 (0.76–6.57)	0.94
BDE-201	75	0.2	0.96 (0.61)	1.06 (0.20–2.46)	0.65
BDE-206	65	1.1–1.2	4.52 (5.82)	2.91 (1.29–23.0)	0.40
BDE-208	65	1.0	3.36 (5.58)	1.50 (0.99–21.6)	0.34
Σ BFRs	100	—	79.5 (154)	43.0 (1.81–96.3)	0.67

Abbreviations: LOD, limit of detection. fw, fresh weight. ICC, intraclass correlation coefficient. TEQ, the World Health Organization Toxic Equivalent values.
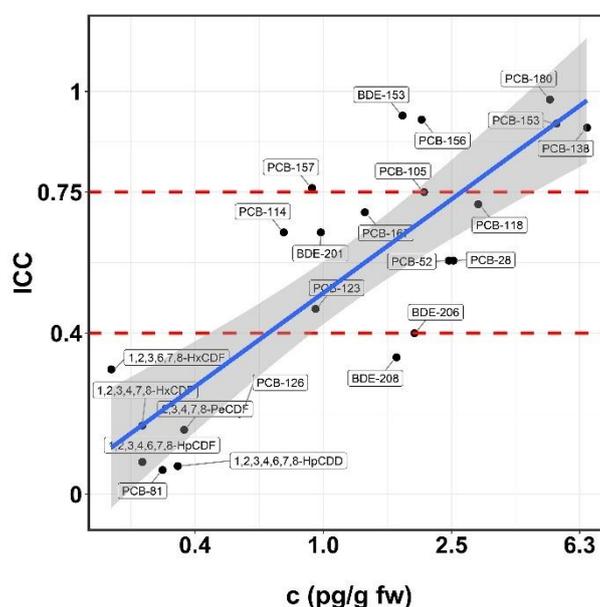
Fig. 7.4 Correlation of the ICCs and concentrations of POPs with DF > 60%.

Table 7.7 compares the TH and element levels in the peri-insertion, mid-disc and periphery regions of human basal plate. The concentrations of T_4, T_3, As, Cr, Hg, Pb, Se, Fe, Ca, K, Mg, Na, Al, Σ heavy metals, and Σ elements were similar across the three regions. rT_3 ($p < 0.05$), Cd ($p < 0.05$), Zn ($p < 0.05$), Mn ($p < 0.05$), and Cu ($p < 0.05$) showed significant differences. Their levels in the periphery regions were 27.6%, 24.7%, 5.43%, 38.9%, and 5.32% lower than in peri-insertion regions, respectively.
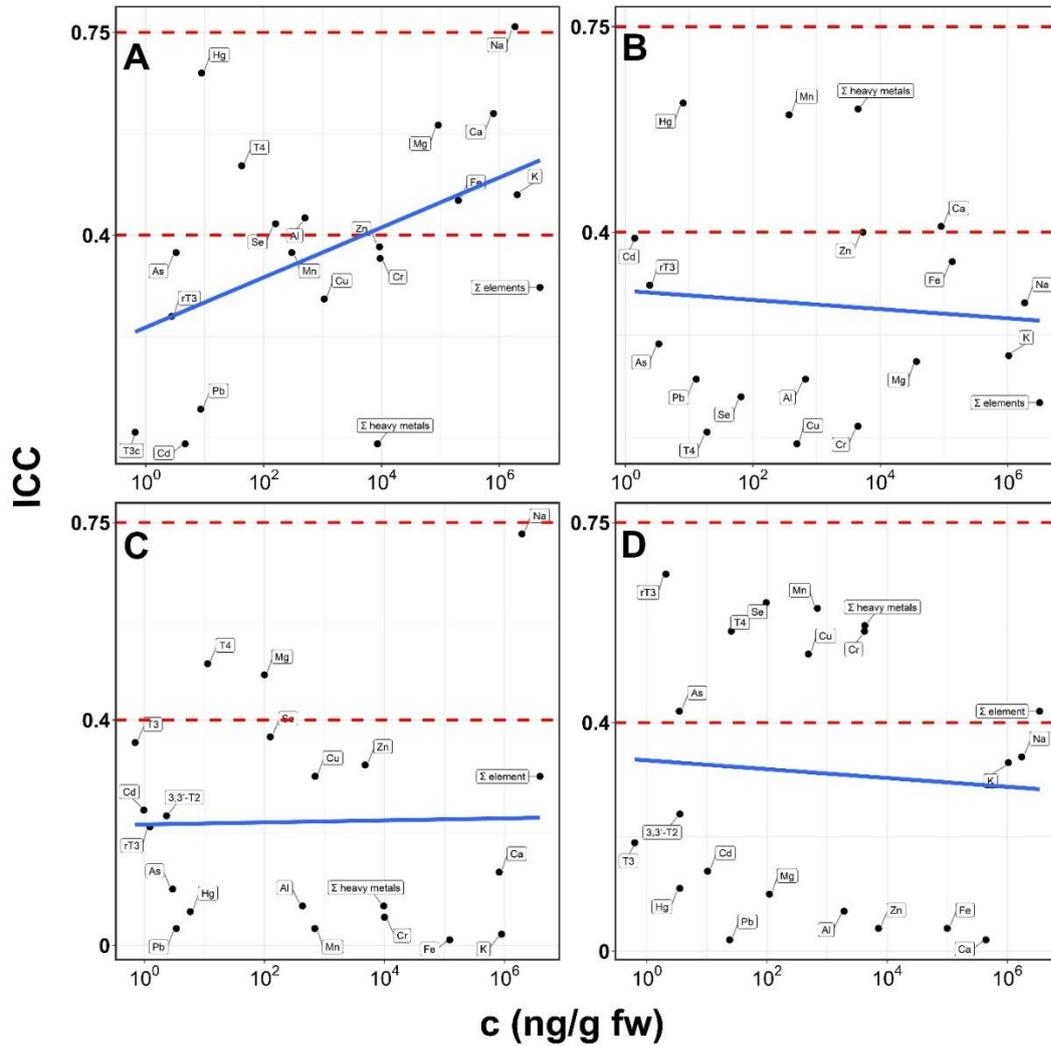
Fig. 7.5 Correlation of the ICCs and concentrations of THs and elements measured in Human placenta (A), Human umbilical cord (B), Porcine placenta (C), and Porcine umbilical cord (D). Concentrations were ln-transformed.

Table 7.8 shows the levels of THs and elements in these regions of porcine placenta. T_4, T_3, rT_3, 3,3'-T_2, Hg, Pb, Se, Zn, Mn, Cu, Fe, Ca, K, Mg, Na and Σ elements were similar across the different regions. As ($p < 0.05$), Cd ($p < 0.05$), Cr ($p < 0.05$), Al ($p < 0.05$) and Σ heavy metals ($p < 0.05$) showed significant differences. Their levels in the periphery regions were 4.12, 7.10, 2.64, 1.80 and 6.80 times higher compared with the peri-insertion region, respectively.

Table 7.6 Descriptive statistics of POPs with detection frequencies < 50% in human placenta

POPs	N (%)	LOD (pg/g)	mean (SD) (pg/g fw)	median (range) (pg/g fw)
PCDD/Fs				
1,2,3,4,7,8-HxCDD	10 (50)	0.001–0.006	0.03 (0.01)	0.03 (0.03–0.05)
1,2,3,6,7,8-HxCDD	10 (50)	0.001–0.007	0.03 (0.01)	0.03 (0.02–0.05)
2,3,7,8-TCDD	0	0.001-0.007	—	—
1,2,3,7,8-PeCDD	4 (20)	0.001–0.007	0.07 (0.03)	0.06 (0.04–0.11)
1,2,3,7,8,9-HxCDD	0	0.001–0.007	—	—
1,2,3,4,6,7,8,9-OCDD	1 (5)	0.29–0.42	0.35	—
2,3,7,8-TCDF	1 (5)	0.001–0.005	0.03	—
1,2,3,7,8-PeCDF	1 (5)	0.001–0.003	0.02	—
1,2,3,7,8,9-HxCDF	0	0.001–0.005	—	—
2,3,4,6,7,8-HxCDF	0	0.01–0.03	—	—
1,2,3,4,7,8,9-HpCDF	0	0.02–0.03	—	—
1,2,3,4,6,7,8,9-OCDF	1 (5)	0.06–0.08	0.25	—
PCBs				
PCB-77	10 (50)	0.3–0.5	0.84 (0.37)	0.81 (0.40–1.40)
PCB-101	3 (15)	6.4–9.3	9.86 (2.53)	10.4 (7.12–12.1)
PCB-169	1 (5)	≤ 0.1	0.07	—
PCB-189	6 (30)	0.4–0.5	1.05 (0.44)	1.23 (0.43–1.46)
PBDEs				
BDE-7	0	≤ 0.02	—	—
BDE-10	0	≤0.03	—	—
BDE-15	9 (45)	0.6–0.9	0.95 (0.31)	0.84 (0.70–1.63)
BDE-17	3 (15)	≤ 0.1	0.12 (0.06)	0.10 (0.07–0.19)
BDE-28	10 (50)	≤ 0.5	0.87 (0.32)	0.82 (0.55–1.61)
BDE-30	0	0.01–0.04	—	—
BDE-47	1 (5)	14.2–20.7	17.6	—
BDE-49	1 (5)	0.4–0.5	1.08	—

BDE-66	2 (10)	0.3–0.5	0.50 (0.03)	0.50 (0.48–0.52)
BDE-71	4 (20)	≤ 0.03	0.22 (0.05)	0.23 (0.14–0.26)
BDE-77	0	≤ 0.03	—	—
BDE-85	3 (15)	0.1	0.32 (0.30)	0.16 (0.14–0.67)
BDE-99	1 (5)	4.5–6.5	9.95	—
BDE-100	1 (5)	1.1–1.7	2.13	—
BDE-119	1 (5)	0.01–0.04	0.18	—
BDE-126	0	0.01–0.05	—	—
BDE-138	0	0.01–0.06	—	—
BDE-139	0	0.01–0.05	—	—
BDE-140	0	0.01–0.05	—	—
BDE-154	9 (45)	0.2–0.4	0.72 (0.33)	0.61 (0.43–1.53)
BDE-156	0	0.01–0.05	—	—
BDE-171	2 (10)	0.04–0.41	1.65 (0.41)	1.65 (1.36–1.94)
BDE-180	0	0.05–0.4	—	—
BDE-183	9 (45)	0.2–2.5	25.2 (6.13)	27.1 (9.82–29.5)
BDE-184	0	0.03–0.37	—	—
BDE-191	0	0.03–0.34	—	—
BDE-196	9 (45)	0.4–0.5	3.75 (0.72)	3.68 (2.45–4.80)
BDE-197	10 (50)	0.9–1.3	8.12 (2.42)	8.69 (2.89–11.2)
BDE-203	9 (45)	0.5–0.7	2.87 (0.69)	2.75 (2.17–4.18)
BDE-204	1 (5)	0.02–0.17	1.09	—
BDE-205	0	0.05–0.53	—	—
BDE-207	10 (50)	2.1–3.0	9.64 (8.49)	7.16 (3.66–33.0)
BDE-209	9 (45)	15.8–21.4	98.0 (183)	34.1 (22.7–586)
HBB	5 (25)	0.8–1.2	1.30 (0.33)	1.15 (1.01–1.80)
BB-153	3 (15)	0.01–0.08	0.76 (0.07)	0.75 (0.69–0.83)

Abbreviations: LOD, limit of detection. fw, fresh weight. HBB, hexabromobenzene. BB-153, 2,2',4,4',5,5'-hexabromobiphenyl.

Table 7.7 Comparison of TH and element levels among peri-insertion, mid-disc and periphery regions of the basal plate of human placenta.

	Peri-insertion[a]	Mid-disc[a]	Periphery[a]	p-value[b]
T_4 (ng/g)	41.1 (37.9–44.9)	42.1 (38.7–45.8)	44.6 (41.0–51.2)	0.192
T_3 (ng/g)	0.59 (0.48–0.75)	0.65 (0.52–0.83)	0.68 (0.55–0.78)	0.377
rT_3 (ng/g)	3.77 (2.97–3.98)	2.90 (1.79–3.83)	2.73 (1.43–3.18)	< 0.05
As (ng/g)	2.29 (1.47–4.97)	2.71 (1.50–8.42)	2.14 (1.39–3.96)	0.455
Cd (ng/g)	4.45 (3.09–5.48)	4.71 (3.96–5.00)	3.35 (2.43–4.48)	< 0.05
Cr (µg/g)	4.87 (2.59–14.8)	7.03 (3.42–29.8)	4.28 (2.50–11.2)	0.363
Hg (ng/g)	6.75 (2.64–14.2)	8.09 (2.91–11.8)	3.23 (1.76–14.3)	0.505
Pb (ng/g)	7.67 (5.49–12.6)	7.67 (5.84–12.8)	8.17 (5.11–12.3)	0.812
Se (µg/g)	0.16 (0.15–0.17)	0.16 (0.15–0.18)	0.16 (0.15–0.18)	0.747
Zn (µg/g)	8.80 (8.31–9.45)	8.80 (8.39–9.39)	8.33 (7.95–8.96)	< 0.05
Mn (µg/g)	0.18 (0.09–0.41)	0.18 (0.10–0.87)	0.11 (0.09–0.17)	< 0.05
Cu (µg/g)	0.94 (0.81–1.03)	1.00 (0.88–1.22)	0.89 (0.80–0.97)	< 0.05
Fe (mg/g)	0.19 (0.14–0.27)	0.18 (0.14–0.34)	0.20 (0.14–0.23)	0.501
Ca (mg/g)	0.52 (0.24–1.06)	0.45 (0.21–1.14)	0.72 (0.41–0.89)	0.563
K (mg/g)	1.97 (1.86–2.11)	1.97 (1.86–2.09)	1.92 (1.78–2.09)	0.313
Mg (µg/g)	81.1 (75.5–89.8)	79.3 (75.9–89.4)	75.9 (71.6–87.9)	0.162
Na (mg/g)	1.83 (1.71–2.00)	1.92 (1.74–2.01)	1.85 (1.63–2.01)	0.522
Al (µg/g)	0.39 (0.26–0.67)	0.41 (0.26–0.51)	0.36 (0.27–0.48)	0.765
Σ heavy metals (µg/g)[c]	4.80 (2.39–14.8)	7.05 (3.44–29.8)	3.99 (1.23–11.1)	0.237
Σ elements (mg/g)[d]	4.89 (4.47–5.53)	4.77 (4.31–6.17)	4.80 (4.55–5.18)	0.520

[a]Median (25th–75th percentiles). [b]The difference of TH and element levels between peri-insertion, mid-disc, and periphery regions were compared using Kruskal-Wallis test. The different regions were divided according to the distance from the spot to the center of the placenta, < 2 cm for peri-insertion, 2–4 cm for mid-disc, and > 4 cm for periphery. [c]The sum of As, Cd, Cr, Hg, and Pb. [d]The sum of all the elements measured.

Table 7.8 Comparison of TH and element levels among peri-insertion, mid-disc and periphery regions of the basal plate of porcine placenta.

	Peri-insertion[a]	Mid-disc[a]	Periphery[a]	p-value[b]
T_4 (ng/g)	11.5 (6.52–17.2)	7.89 (6.17–12.8)	10.0 (7.55–16.3)	0.399
T_3 (ng/g)	0.62 (0.41–0.80)	0.43 (0.28–0.70)	0.68 (0.32–1.10)	0.360
rT_3 (ng/g)	1.20 (1.07–1.30)	1.05 (0.94–1.42)	1.21 (1.10–1.36)	0.468
3,3'-T_2 (ng/g)	1.99 (1.52–2.57)	2.32 (1.79–3.64)	1.67 (1.16–3.69)	0.494
As (ng/g)	1.12 (0.89–1.19)	2.27 (0.98–4.46)	4.61 (1.81–7.37)	< 0.05
Cd (ng/g)	0.32 (0.26–0.34)	0.77 (0.66–1.18)	0.89 (0.39–1.91)	< 0.05
Cr (µg/g)	2.31 (0.58–3.58)	4.91 (1.68–14.6)	16.4 (5.97–29.1)	< 0.05
Hg (ng/g)	3.44 (1.95–4.25)	4.75 (3.54–8.17)	4.89 (3.87–6.13)	0.228
Pb (ng/g)	3.04 (2.42–3.44)	2.84 (2.36–3.77)	3.60 (3.07–6.34)	0.488
Se (µg/g)	0.11 (0.09–0.18)	0.11 (0.10–0.13)	0.12 (0.11–0.14)	0.712
Zn (µg/g)	4.41 (4.04–5.07)	5.18 (4.82–5.49)	4.77 (4.09–5.14)	0.130
Mn (µg/g)	0.36 (0.33–0.61)	0.62 (0.45–0.96)	0.95 (0.47–1.56)	0.082
Cu (µg/g)	0.70 (0.55–0.81)	0.78 (0.63–0.92)	0.89 (0.70–0.96)	0.362
Fe (mg/g)	0.05 (0.03–0.09)	0.09 (0.04–0.17)	0.13 (0.08–0.18)	0.108
Ca (mg/g)	0.81 (0.63–0.96)	0.80 (0.71–0.94)	0.78 (0.76–0.83)	0.968
K (mg/g)	0.91 (0.80–1.01)	0.89 (0.86–0.99)	0.87 (0.77–0.91)	0.527
Mg (µg/g)	98.1 (90.5–124)	93.7 (82.1–109)	90.1 (87.1–141)	0.408
Na (mg/g)	1.84 (1.77–2.04)	2.12 (1.83–2.19)	1.96 (1.69–2.06)	0.204
Al (µg/g)	0.20 (0.16–0.26)	0.38 (0.27–0.55)	0.36 (0.28–0.53)	< 0.05
Σ heavy metals (µg/g)[c]	2.41 (0.58–3.59)	4.92 (1.68–14.6)	16.4 (5.98–29.1)	< 0.05
Σ elements (mg/g)[d]	3.68 (3.60–3.95)	4.07 (3.71–4.24)	3.91 (3.77–3.98)	0.381

[a]Median (25th–75th percentiles). [b]The difference of TH and element levels between peri-insertion, mid-disc, and periphery regions were compared using Kruskal-Wallis test. The different regions were divided according to the distance from the spot to the center of the placenta, < 2 cm for peri-insertion, 2–4 cm for mid-disc, and > 4 cm for periphery. [c]The sum of As, Cd, Cr, Hg, and Pb. [d]The sum of all the elements measured.

7.4 Discussion

7.4.1 Chemical analysis in placentome

The TH levels in human basal plate of placenta reported here were similar to previous findings using the entire human placenta [37]. De Moraes *et* al. observed that the levels of Ca, Fe, Cu, and Zn in the placenta of adult mothers were 701, 85.3, 0.8, and 12.7 μg/g fw, respectively, while their levels in the placenta of teenage mothers were 818, 103, 0.6, and 13.8 μg/g fw, respectively [146]. A previous review reported that the average levels of As, Cd, Hg, Pb, Se, Zn, Mn, Cu, Ca, K, Mg, and Al in human placenta were 0.006, 0.004, 0.008, 0.034, 0.2, 10, 0.08, 0.9, 770, 1685, 100, and 0.25 μg/g fw, respectively [243, 244]. These values were in line with our results although the authors did not indicate the sampling positions. However, the concentrations of Cr (9.46 vs. 0.03 μg/g fw) and Fe (200 vs. 69 μg/g fw) were higher in this study, probably because of the differences in exposure level, blood content, and potential contamination during sampling. The placental levels of BFRs measured here were similar to previous reports in Europe while PCBs were higher in our study [245], which could be due to the differences in exposure level and sample size. NE in cord blood measured here was about 10 times lower compared with previous study while DA was higher [246], which could be due to the differences in the modes of delivery [247] and demographic characteristics such as smoking [248]. Monoamines were < LOD in all placenta samples, probably because of the high monoamine oxidase activity in placenta [249].

7.4.2 Trans-placental transfer of THs, elements, and monoamines

In placenta, the fetal blood vessels locate close to the surface of the expanding trophoblastic villi to approximate with maternal vessels [135]. This interface allows the exchange of chemicals between maternal and fetal circulations. The following mechanisms are potentially involved: (1) Passive diffusion [136]; (2) Transporting proteins, i.e., transthyretin (TTR) [70], metallothionein [250], and transferrin [251]; (3) Local metabolism, i.e., deiodination [252], oxidation [253, 254], and sulfotransferation [255]; (4) Lipid content [145] and blood content [148]. Environmental exposure [70, 255-257] and maternal physiological conditions [146, 153, 258] can influence this process by interfering with the expression of transporters and enzymes.

Only a few previous studies examined the transfer of certain elements in human placentome [146, 148]. Our results show that TH and element behave differently in human and porcine placentome. Lower permeability was observed in human placentome. This is probably due to the structural differences between human and porcine placentas. Human placenta is hemochorial in which the placental trophoblast is in direct contact with maternal blood, while pig placenta is epitheliochorial in which the trophoblast is apposed to the epithelium of the uterus [259]. The ample fetal villi section is rich in umbilical arteries and veins. The chemical distribution variability in this section of placenta mainly results from the differences in blood content [148].

Trans-placental delivery of THs is regulated by plasma membrane transporters, iodothyronine deiodinases and proteins within trophoblast cells [91]. While T_4 was concentrated in basal plate of human placentome, T_4, rT_3 and $3,3'-T_2$ showed higher levels in umbilical cord of porcine tissues, indicating higher expression of TTR and deiodinases in porcine placentome. The placenta is known to block the transfer of Cu and Cd, but is a weak barrier for Pb and Mn [243, 260, 261], which explains the higher Cu and Cd levels but lower Pb and Mn levels in human placenta here. This has potential clinical implications because Cd exposure could reduce the essential Ca and Zn transfer to the fetus by competitively binding with metallothionein [244], while perinatal Pb exposure may contribute to the etiologies of autism spectrum disorder [137]. Previous studies observed higher levels of Ca and Zn concentrations in human placenta than in fetal portion [146, 148], which agrees well with our study. However, de Moraes *et* al. found lower Ca level in placenta of adult mother [146], suggesting an age-dependent expression of Ca^{2+} transporter/channel. Human placenta could partially block the As transfer [244]. However, we did not find significant difference of As level between human placenta and cord. Fe from maternal circulation is transferred to the fetus by binding with transferrin along a unidirectional pathway [262], leading to lower Fe level in human umbilical cord. An active transfer of Hg in placenta was suggested previously [263], which was not found in this study, probably due to the high variation of Hg concentration in our data. Some other studies assessed the transplacental transfer of elements by comparing their levels in maternal plasma and cord blood, which revealed higher levels of Cd, Cu, Zn, and Se

in maternal plasma [261, 262]. These results are in line with our study.

Although 9 monoamines were targeted for analysis, only NE and DA were quantified in cord blood while the other congeners were < LOD in all samples. These results suggest that monoamines may not be transferred and the NE and DA in cord blood might be of fetal origin.

7.4.3 Distribution variability of chemicals in placenta

The poor reliability of certain THs and elements in placentome observed here is consistent with previous reports [148, 149]. rT_3, Cd, Zn, Mn, Cu in human basal plate, and As, Cd, Cr, Al, and Σ heavy metals in porcine basal plate showed significant differences among the regions. These results corresponded well with their low ICC values, although we did not observe regional difference for some other chemicals with low ICC such as Pb in human basal plate. Besides, the significant differences of biological variation of certain THs compared with measurement variation also confirmed their low ICCs. To our knowledge, only one study assessed the regional distributions of chemicals in placenta, in which higher Fe level was found in the central region of human placenta. The authors attributed to the regional differences in blood flow [148]. In this study Fe concentration was similar among these regions (p=0.501), indicating similar blood content among the spot samples. Manci et al. also observed higher Ca concentration in the periphery region [148]. In our study, Ca level in the periphery was higher but not significant. Besides, we observed higher concentrations of As, Cd, Cr, Al, and Σ heavy metals in the periphery region of porcine placenta. These results indicate that the regional differences might be due to the regional expressions of transporting proteins and metabolic enzymes. Further assessment of the regional distributions of the proteins responsible for the transporting and metabolism of THs and elements could better explain the results here.

Exposures to the most congeners of PCBs and PBDEs had a high degree of reliability in placenta in our study, with ICCs of 0.40–0.98 except PCB-81, 126, and BDE-208. However, all the PCDD/Fs and their WHO-TEQ values showed low reliability. These findings suggest different transfer mechanisms of PCDD/Fs with PCBs and PBDEs. For example, PCDD/Fs may induce the expression of cytochrome P450 enzymes which may interfere with their metabolism and clearance [264], leading to larger variances.

Taken together, our results indicate that substantial exposure misclassification may happen

in epidemiological studies when placental TH and element exposures are classified using a single spot placenta or umbilical cord samples. To avoid potential sampling bias, previous studies advised to collect the entire placenta or clearly define the location of the sampling sites [93]. However, due to the differences in placental distribution characteristics, we propose to consider different sampling procedures. Random sampling might be sufficient for PCB and PBDE analyses due to their good reliabilities. However, collecting the entire placenta or pooled samples from different regions seems unavoidable for most THs, elements and PCDD/Fs. Some other chemicals such as monoamines in cord blood, however, may originate from the fetus. Placenta is not an appropriate source for such biomarkers to assess maternal situations.

7.4.4 Strengths and limitations

This study has several unique strengths: (1) As many as 105 chemicals (THs, elements, POPs, and monoamines) were analyzed in this study, which can represent a profile of the most prominent constituents in intrauterine environment; (2) We comprehensively assessed the regional distribution and trans-placental transport properties of the chemicals. This could help to optimize the sampling strategy, reduce sampling bias, and better indicate the disease etiology and progression; (3) Since more representative data was obtained in this study, the concentrations, transplacental potencies, and distribution variances obtained here may better reveal the real situations. Our study also has certain limitations. For example, the number of tissues is limited, which may reduce the statistical power. A previous study observed differences of the element levels between maternal and fetal placenta [146]. TH and elemental analyses in this study were performed in basal plate although the levels here were similar to previous reports with entire placenta.

7.5 Conclusion

In summary, we investigated the placental distribution and transfer of various chemicals. We observed different transplacental potencies for different chemicals. We found heterogeneous distributions for certain THs, elements, and PCDD/Fs, while PCBs and PBDEs showed high reliability. Our results suggest different modes of placental distribution and transfer, and highlight the challenges of assessing intrauterine exposures because of the

sampling bias. Different sampling strategies should be applied according to their distribution variation. Further studies regarding the regional differences of transporting proteins and enzymes are warranted. Finally, the findings in this study should be confirmed with more samples with the inclusion of other sections of placenta.

Chapter 8 Determination of 3-T$_1$AM in blood and tissues using LC-MS/MS

Abstract

3-T$_1$AM has been proposed to be a novel chemical messenger because of the following properties: (1) 3-T$_1$AM exists in the brain of rodent; (2) 3-T$_1$AM is a multitarget ligand and can interact with TAAR1, certain aminergic receptors, mitochondrial proteins, apoB-100, and transient receptor potential channels; (3) significant hypothermia and bradycardia were observed in mouse following administration of 3-T$_1$AM; and (4) 3-T$_1$AM can reduce contractile performance and heart rate in the isolated perfused rat heart. However, discrepancies have been reported in previous studies regarding the existence of endogenous 3-T$_1$AM. In this chapter, we developed an LC-QqQ-MS method combined with isotope dilution for the quantification of 3-T$_1$AM in mouse blood and tissues. We observed significant decrease in 3-T$_1$AM/^{13}C$_6$-T$_1$AM concentrations after incubating with fetal bovine serum (FBS). The half-lives of 3-T$_1$AM/^{13}C$_6$-T$_1$AM ranged from 5 to 157 min, depending on the initial concentration of 3-T$_1$AM/^{13}C$_6$-T$_1$AM (1 to 1000 ng/mL). 3-T$_1$AM/^{13}C$_6$-T$_1$AM showed shorter half-lives when incubated in higher concentrations of FBS. 3-T$_1$AM and ^{13}C$_6$-T$_1$AM showed similar dynamic parameters, indicating ^{13}C$_6$-T$_1$AM a proper internal standard for 3-T$_1$AM. For sample cleanup, we employed solid-liquid extraction, liquid-liquid extraction, and solid-phase extraction. After optimization, we observed 3-T$_1$AM in mouse liver following administration with NAc-T$_1$AM and OAc-T$_1$AM. These results suggest that with further modifications, our approach can be hopefully used for the determination of endogenous 3-T$_1$AM.

8.1 Introduction

As described in Chapter 2, we developed UPLC-Q-TOF-MS and HPLC-QqQ-MS methods for the quantification of THs in various biomatrices. 3-T$_1$AM was also targeted in these methods, which, however, was always < LOD. This indicates that the sensitivity of these methods is not sufficient for 3-T$_1$AM analysis. Scanlan *et* al. and Saba *et* al. have validated a method using LC-MS/MS for 3-T$_1$AM analysis in tissues and blood [18, 45]. In this chapter we first tried to reproduce the method reported by Scanlan and Saba, and further assessed the recoveries of 3-T$_1$AM in serum.

8.2 Materials and methods

8.2.1 Materials

Fetal bovine serum (FBS), Dulbecco's minimum essential medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bond Elut Certify SPE Large Reservoir Capacity cartridges (300 mg/10 mL, 120 μm), and Bond Elut Certify SPE cartridges (50 mg/3 mL) were obtained from Agilent technologies (Santa Clara, CA, USA). Protease from Streptomyces griseus, phosphate buffered saline, and potassium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Serum, liver, kidney, skeletal muscle, stomach, intestine, white adipose tissue, lung, heart, and brain of female CD-1 mice (4-month old, weight: 40–45 g) were provided by Dr. Manuel Miller and Dr. Angelika Scheideler. Liver samples of C57BL/6J mice were provided by Dr. Sonja Charlotte Schriever and Dr. Paul Pfluger. Liver samples from mice dosed with NAc-T$_1$AM or OAc-T$_1$AM were obtained from Prof. Jens Mittag. C57BL/6J male mice at the age of 8-12 weeks and housed in single cages at an ambient

temperature of 22°C with a 12h dark/light cycle at the GTH University of Lübeck or the KMW Animal Facility of the Karolinska Institute Stockholm. The animals were injected once per day with either NAc-T_1AM or OAc-T_1AM at 5mg/kg bodyweight i.p. dissolved in 60%DMSO in PBS.

8.2.2 HPLC-MS/MS

For the quantification of 3-T_1AM with HPLC-QqQ-MS, we monitored four transitions to avoid false identification. The optimized MS/MS parameters are shown in Table 8.1. The other parameters were the same with those reported in Chapter 4.

8.2.3 Experimental procedures

(1) Recovery of 3-T_1AM in serum and tissue

Five hundred micro litter of DMEM supplemented with 10% FBS was employed as the incubation buffer. 3-T_1AM and $^{13}C_6$-T_1AM were spiked to reach concentration from 1 ng/mL (2.8 nM) to 1000 ng/mL (2800 nM). The mixture was incubated at 37 °C for 0, 20, 40, 60, 120, and 180 min. At each time point, samples were extracted using a liquid-liquid approach: 1 mL of methyl tert-butyl ether (MTBE) was added, vortexed vigorously, and centrifuged at 7000 rpm for 10 min. The organic supernantant was decanned into a new tube. The extraction process was repeated twice more and the extracts were combined. The combined organic solution was evaporated to dryness under N_2 at 40°C. Afterwards, the sample was reconsistuted in MeOH:0.1 M HCl (1:1, v/v) and measured with HPLC-QqQ-MS. The recoveries of 3-T_1AM and $^{13}C_6$-T_1AM were regressed using the first-order kinetics.

In order to examine the influence of the concentration of FBS in the incubation solution on the decrease dynamics of 3-T_1AM and $^{13}C_6$-T_1AM, we repreated the experiment by using DMEM/50% FBS and 100% FBS, respectively. To investigate the influence of the pH value, we adjusted the pH value of DMEM/10% FBS to 10 using 1M NaOH. Afterwards, 100 ng/mL was spiked, and kept at 37°C for 60 min. Similarly, to examine the effect of protein deproteinization, the buffer was kept at 100°C for 30 min. Afterwards, 100 ng/mL was spiked, and kept at 37°C for 60 min.

Similar processes were also applied for mouse liver homogenate. 500 mg liver from CD-1 mouse (female, 4 weeks) were homogenized using ultrasonication, 3-T_1AM and $^{13}C_6$-T_1AM were spiked and processed as described above.

Table 8.1 Optimized MS/MS parameters for the quantification of 3-T$_1$AM.

	Precursor ion	Product ion	Dwell	Fragmentor	CE	CAV
3-T$_1$AM	356	339	50	118	24	2
	356	212	50	118	40	2
	356	195	50	118	44	2
	356	165	50	118	28	2
^{13}C$_6$-T$_1$AM	362	345	50	118	36	2
	362	218	50	118	60	2
	362	201	50	118	72	2
	362	171	50	118	28	2

Abbreviations: CE, collision energy; CAV, cell acceleration voltage.

(2) Quantification of 3-T$_1$AM

Initially, we followed the sample cleanup procedures described by Scanlan *et* al. for 3-T$_1$AM analysis (shown in Fig. 1.2 (A)) or Saba et al. (B). Afterwards, we modified the procedure B to release the binding of 3-T$_1$AM from apo-B100 with 3 approaches:

(i) 1 g of mouse liver was homogenized after adding 0.5 mL 1×phosphate buffer (154 mL NaCl, NaH$_2$PO$_4$, pH 7.4) at 4°C. Afterwards, 1 mL 0.1 g/mL trichloroacetic acid (TCA) solution was added and kept on ice for 1 h. The further processes were the same with procedure B.

(ii) 500 mg mouse liver was homogenized after adding 0.5 mL 1×phosphate buffer (154 mL NaCl, NaH$_2$PO$_4$, pH 7.4) at 4°C. Afterwards, 1 mL of 100 mg/mL protease solution was added. After gentle shake, the sample was kept at 37°C for 24 h. 0.5 mL MeOH was added and kept on ice for 1 h after vortex, then internal standard was spiked. The sample was further processed with Bond Elute Certify cartridges.

(iii) 500 mg mouse liver was homogenized after adding 0.5 mL 1×phosphate buffer (154 mL NaCl, NaH$_2$PO$_4$, pH 7.4) at 4°C. Afterwards, 1 mL 8 M urea was added and kept at 50 °C for 1 h. The pH of the sample was adjusted with 12 M HCl. Then the sample was processed with Bond Elute Certify cartridges.

8.3 Results and discussion

8.3.1 HPLC-QqQ-MS

For the calculation of LODs and LOQs, the standard solution (0.5 pg/µL) were repeatedly measured for 8 times. Three times the SD provides LOD while 10 times the SD provides LOQ. To improve sensitivity for 3-T_1AM, we modified the analytical approach as described in Chapter 6. Water (A) and ACN (B) were used as mobile phases. We examined the effects of different additives. Compared with adding 0.1% formic acid, adding 5 µM ammonium formate in mobile phases provided improved signal intensity for 3-T_1AM (6 times higher). However, similar LOD/LOQs of THs were observed in mobile phases spiked with both additives (as shown in Table 8.2).

Table 8.2 LODs and LOQs of THs in mobile phases added with 0.1% formic acid or 5 µM ammonium formate.

	LOD (pg/µL)	LOQ (pg/µL)	LOD (pg/µL)	LOQ (pg/µL)
	0.1% formic acid		5 µM ammonium formate	
3-T_1AM	0.04	0.13	0.04	0.12
3,5-T_2	0.15	0.49	0.14	0.47
3,3'-T_2	0.13	0.45	0.11	0.36
T_3	0.06	0.21	0.10	0.34
rT_3	0.11	0.37	0.08	0.28
T_4	0.18	0.60	0.11	0.37

8.3.2 Recovery of 3-T_1AM in serum and tissue homogenate

As shown in Fig. 8.1, decreasing recoveries of 3-T_1AM and $^{13}C_6$-T_1AM were observed when incubating with DMEM/10% FBS. After 60 min, only 2% of the compounds were recovered.

As shown in Table 8.3, 3-T_1AM and $^{13}C_6$-T_1AM behaved exactly the same. Lorenzini *et*

al. observed that D_4-T_1AM behaved differently with 3-T_1AM [40]. This proved that $^{13}C_6$-T_1AM is a better internal standard for 3-T_1AM analysis. The half-lives of 3-T_1AM and $^{13}C_6$-T_1AM were 5–157 min when the initial concentrations of the substrates ranged from 1 to 1000 ng/mL. Besides, when incubated with higher concentrations of FBS, 3-T_1AM and $^{13}C_6$-T_1AM showed shorter half-lives, as shown in Fig. 8.2.
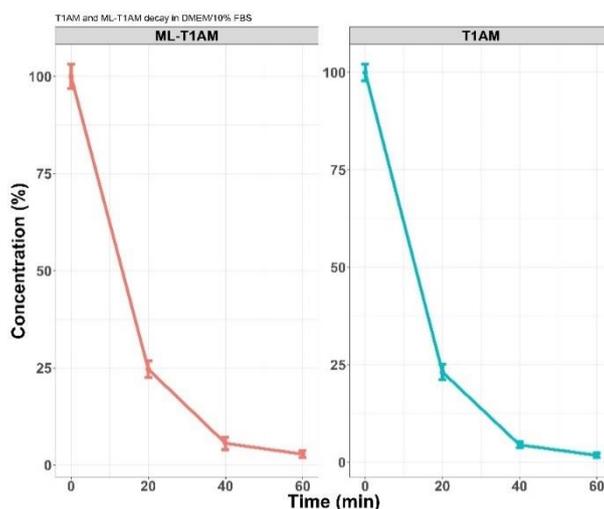

Fig. 8.1 3-T_1AM and $^{13}C_6$-T_1AM were added at time 0 in DMEM/10% FBS (v/v). Data are shown as relative signal.

Table 8.3 Dynamic parameters of the decay of 3-T_1AM and $^{13}C_6$-T_1AM in DMEM/10% FBS[a].

Concentration	3-T_1AM			$^{13}C_6$-T_1AM		
	K (1/min)	Half-life (min)	R^2	K (1/min)	Half-life (min)	R^2
1000 ng/mL	0.044 ± 0.004	157	0.948	0.047 ± 0004	148	0.949
500 ng/mL	0.011 ± 0.006	61.6	0.911	0.012 ± 0.007	59.4	0.907
250 ng/mL	0.033 ± 0.005	21.3	0.990	0.036 ± 0.006	19.3	0.986
100 ng/mL	0.063 ± 0.008	11.1	0.979	0.094 ± 0.006	7.34	0.995
50 ng/mL	0.093 ± 0.011	7.44	0.978	0.151 ± 0.017	4.58	0.977
10 ng/mL	0.147 ± 0.010	4.71	0.991	0.227 ± 0.018	3.05	0.989
5 ng/mL	0.135 ± 0.007	5.15	0.995	0.203 ± 0.014	3.41	0.992

| 1 ng/mL | 0.138 ± 0.005 | 5.04 | 0.997 | 0.203 ± 0.013 | 3.41 | 0.993 |
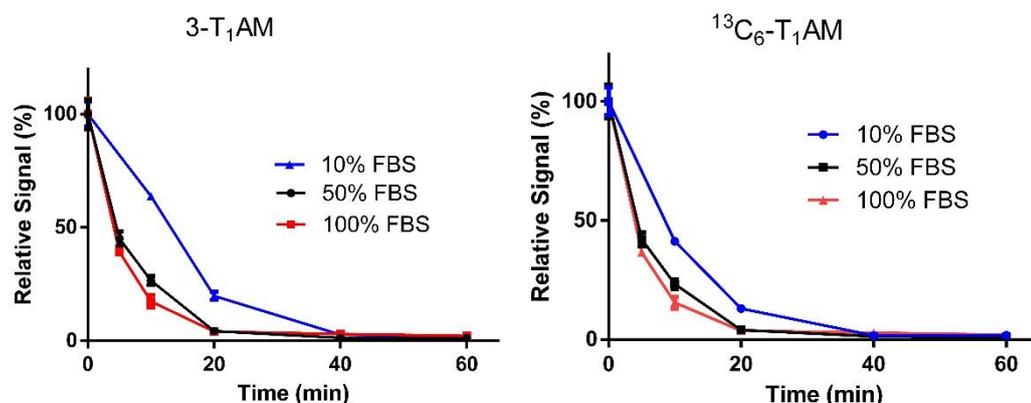
Fig. 8.2 Decrease of 3-T_1AM and $^{13}C_6$-T_1AM when incubated with different levels of FBS.

Fig. 8.3 shows the influence of pH value of the incubation buffer on the decreases of 3-T_1AM and $^{13}C_6$-T_1AM. No significant decrease of T_1AM/ $^{13}C_6$-T_1AM recoveries were observed. This is probably due to the deproteinization at pH 10. Similarly, the recoveries of T_1AM and $^{13}C_6$-T_1AM kept constant after deproteinization with 100°C/30 min (data not shown).
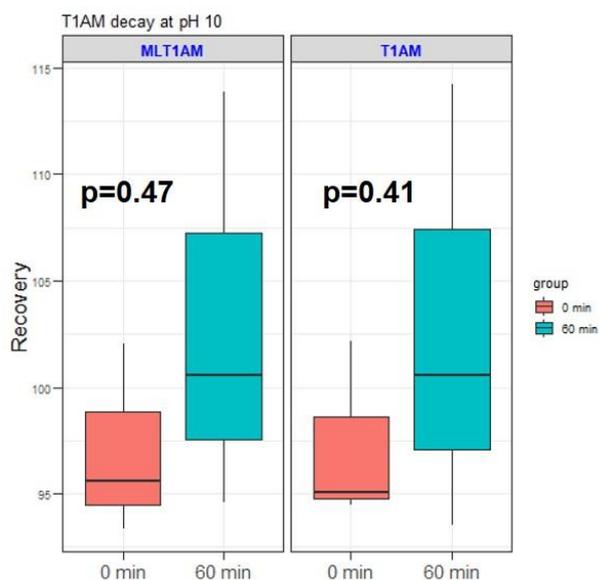
Fig. 8.3 Influence of pH on the decreases of 3-T_1AM and $^{13}C_6$-T_1AM in DMEM/10% FBS.

However, when incubated with mouse liver homogenate, the recoveries kept almost 100% after 60 min. This is probably because the binding protein (ApoB-100) is abundant in serum while is minor expressed in liver (shown in Fig. 8.3).
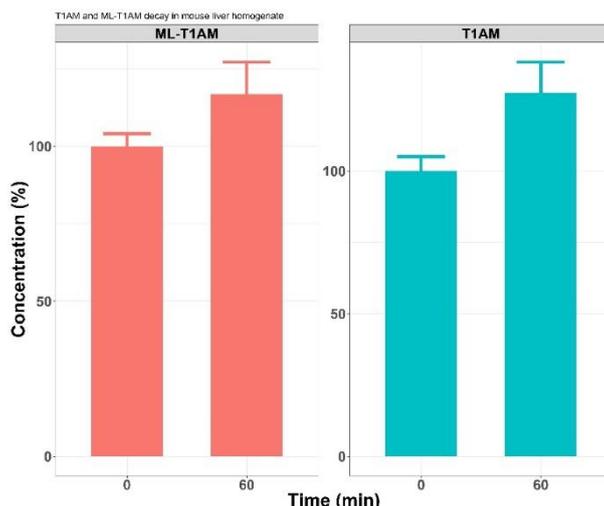
Fig. 8.4 3-T$_1$AM and ^{13}C$_6$-T$_1$AM were added at time 0 in mouse liver homogenate. Data are shown as relative signal.

8.3.3 Analysis of 3-T$_1$AM in mouse liver

Initially, we followed the sample cleanup procedures reported by Scanlan *et* al. and Saba *et* al. (Fig. 1.2) [18, 45]. However, we were not able to reproduce the results. These difficulties and discrepancies are tentatively attributed to the high affinity binding of 3-T$_1$AM to serum Apo-B100 in a Schiff base-like manner [55].

Protein removement with protease or urea were not able to produce a clean peak. In order to break the binding of 3-T$_1$AM with apo-B100, we employed 3 different methods: (1) TCA (0.1 g/mL) which is able to precipitate the proteins and break the Schiff-base binding; (2) Urea which is able to remove proteins from the homogenate; (3) Protease which is able to digest proteins.

As shown in Fig. 8.5, in the sample processed with 0.1 mg/mL TCA, we observed clear peaks of 3 transitions of both 3-T$_1$AM and ^{13}C$_6$-T$_1$AM. This result indicates that this approach was able to release and quantify 3-T$_1$AM. However, we were not able to reproduce the results.
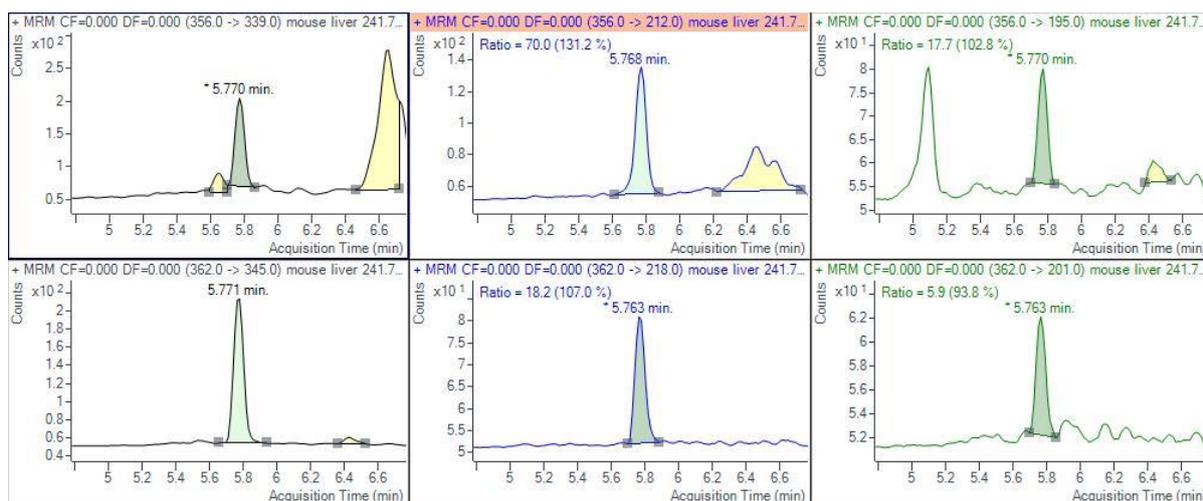
Fig. 8.5 Observed transitions of 3-T_1AM and $^{13}C_6$-T_1AM in mouse liver.

We further measured the concentration of 3-T_1AM in mouse liver that have been dosed with NAc-T_1AM and OAc-T_1AM. As shown in Fig. 8.6, clear peaks of 3-T_1AM were found. This is probably because of the high exposure levels
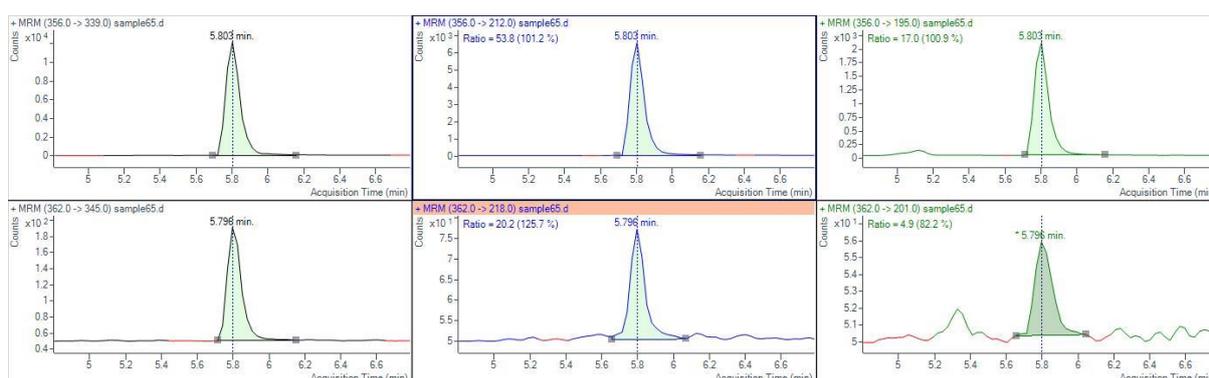

Fig. 8.6 Chromatograms of 3-T_1AM observed in the liver of mouse that has been dosed with NAc-T_1AM.

8.4 Conclusion

We observed the same time-dependent decreases of 3-T_1AM and $^{13}C_6$-T_1AM in FBS, indicating $^{13}C_6$-T_1AM a proper internal standard for 3-T_1AM analysis. We employed different approaches to release and quantify endogenous 3-T_1AM. Further efforts are essential to develop and optimize a reliable method to release 3-T_1AM. Besides, the binding properties of 3-T_1AM with proteins is of critical importance.

Chapter 9 Discussion and outlook

9.1 Discussion

This dissertation focusses on human exposomics of POPs in relation to endogenous biomarkers (THs and monoamines) and metabolic syndrome (GDM and T2D). The major contents of this dissertation are outlined in Fig. 9.1.
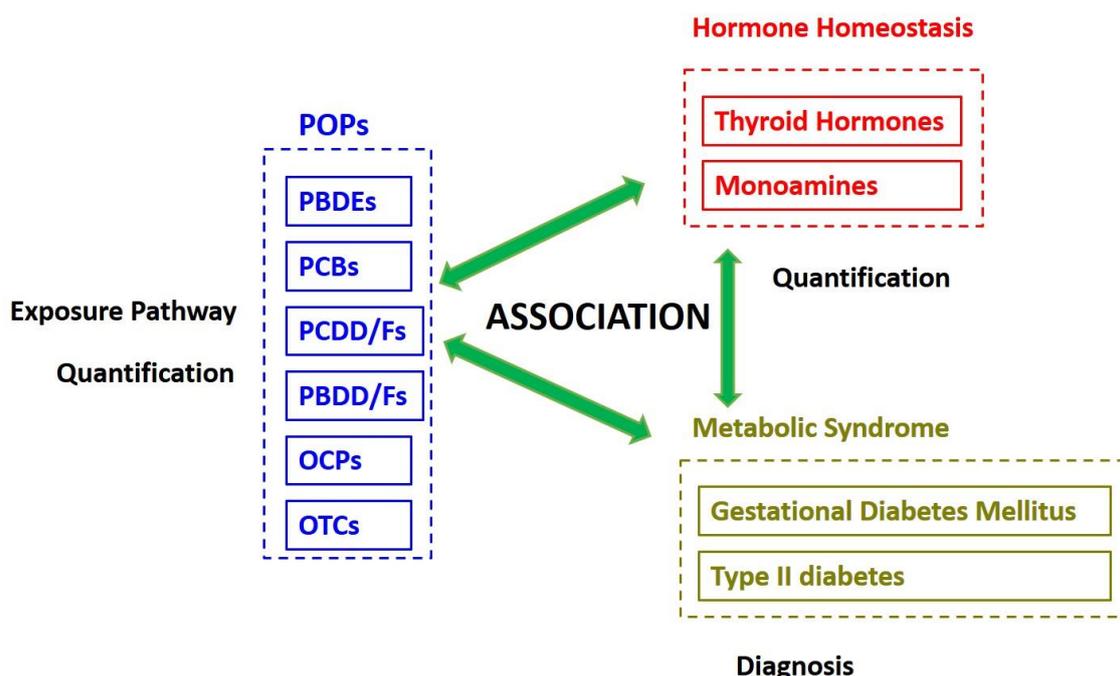

Fig. 9.1 Major contents of this dissertation.

Analytical methods using UPLC-Q-TOF-MS and HPLC-MS/MS were developed and optimized for the quantification of THs. High sensitivity and selectivity of the methods were observed. These methods were applied to determine THs in various biomatrices such as human placenta, human serum, mouse liver, and mouse plasma. The analytical approaches with improved selectivity and sensitivity will allow comprehensive evaluation of TH homeostasis in research of metabolism and effects of environmental contaminant exposures. Besides, the methods were evaluated and validated comprehensively. These validation procedures can be employed by future researchers to evaluate their methods.

The thyroid-disrupting properties of environmental contaminants, including PBDEs, PCBs, PCDD/Fs, PBDD/Fs, OCPs, and OTCs, were examined in this dissertation. The human

exposomics of POPs can be assessed using different samples, which can give different results because of the differences in the contents of lipid, proteins, as well as the sample amount that can be obtained. TH disorder during pregnancy may hamper the fetal development, especially the neurodevelopment. For this purpose, human placenta samples from the Danish EXPORED study, and human breast milk samples from the German LUPE study were collected. Due to the high lipid content, human breast milk provided improved detection frequencies for POPs, which make the models more robust and promising. Different statistical approaches were applied for evaluating the associations of THs with POPs, including single pollutant model, multiple linear regression, principal component analysis, partial least linear regression, and hierarchical clustering. Similar results were observed in both EXPORED and LUPE cohorts, which showed inverse associations between PBDEs and THs. The findings in this dissertation enables to evaluate the human background exposure of POPs and the related health outcomes. Considering the critical physiological roles of THs, the results obtained in this dissertation are of great significance in public health and eco-toxicology.

The potential influence of GDM on the placental THs was evaluated in this dissertation. Samples were from a Chinese birth cohort study. Pearson's Chi-square test was applied with a set of covariates adjusted. The results indicated that THs may not be associated with GDM. However, the statistical power of this analysis was limited because of the small sample size of the case group (n=32). Although no significant associations between GDM and placental THs was found, the results in this dissertation can be helpful for future researchers to design their study on this topic.

Considering the complex mixture of exposures, EWAS targets to obtain comprehensive, quantitative measurements of chemicals in human biospecimens. EWAS requires large sample size which increases the expense and workload. Sample-pooling strategy is a promising approach in EWAS. This procedure was employed in the German CARLA cohort to assess the associations of POP exposure with T2D. The results showed that the exposure burdens of certain POPs were significantly higher in the pooled cases compared with the pooled controls. These findings suggest that sample pooling approach could be a useful explorative tool in spite of the existence of limitations. Finally, sample pooling method needs further development. The

findings could encourage future efforts to develop sample pooling approach as a promising technology in EWAS.

Placentome has been increasingly employed to assess the relationship between environmental exposure and placental programming. Obtaining a representative biospecimen from placentome is crucial considering the potential heterogeneity of chemical distributions. The placental distribution of various chemicals was examined in this dissertation. Different distribution properties were observed for different chemicals. The results suggest different modes of placental distribution, and highlight the challenges of assessing intrauterine exposures because of the sampling bias. Different sampling strategies should be applied according to their distribution variation. Besides, a novel method was developed for cryo-based sampling, sample storage, and subsampling. This method enables large cohorts to obtain reliable biomarkers.

3-T$_1$AM has been reported to be an endogenous metabolite of thyroxine. Both IA and LC-MS/MS methods have been developed. However, significant challenges still exist because discrepancies exist between the results reported in different laboratories. An LC-MS/MS approach was developed and optimized for the quantification of endogenous 3-T$_1$AM. The recoveries of 3-T$_1$AM in serum and tissues were also evaluated. The results indicated that ^{13}C$_6$-T$_1$AM might be a better internal standard compared to D4-T$_1$AM, which has been reported to have certain pitfalls. The method in this dissertation can be used for the quantification of endogenous 3-T$_1$AM and thereby, reveal the physiological properties of 3-T$_1$AM.

9.2 Outlook

Tandem MS is a promising technology for the quantification of THs, and LC-MS has become the method of choice for many laboratories. Quality assurance and quality control are important to avoid mis-identification for THs at low concentration level. For example, there does not exist a commonly accepted rule as to the use of quantification and confirmation transitions and their ratios. Furthermore, the isotope-labelled quantification standards for certain THs (i.e., 3,3'-T$_2$) are not commercially available. Besides, the deuterated internal standard and ^{13}C- labelled internal standard may show different recoveries compared with the

target analytes, which may overestimate or underestimate the concentration.

Different epidemiologic studies reported significant inconsistences on the relationship between POPs and THs, which hampers the assessment of the environmental risks of these chemicals. Possible reasons include presence of different pollutant mixtures, different specimen, different exposure levels, varying timing of sample collection, and different living environment and life styles of populations. Future studies with longitudinal design may better address these problems and provide more evidence.

In circulation, 99.98% of T_4 and 99.7% of T_3 are bound to plasma-binding proteins. The free forms might be more sensitive to the physiological changes and environmental exposure. The lack of specificity, bias, and imprecision are analytical pitfalls [25]. Further studies are warranted to developed sensitive and selective technology to quantify free THs and assess their associations with POP exposure.

Exposome refers to the totality of exposures throughout the lifespan. Exposomic approaches can include all exposures of potential health significance, whether from endogenous or exogenous sources. Exposomic biomonitoring offers an efficient means for characterizing individual exposure profiles. Incorporating the exposome paradigm into biomonitoring promote exposure assessment in many ways. Besides, genetics and epigenetics are more sensitive to the exposures than physiological changes. Genetic and epigenetic epidemiology could be more sensitive tools to investigate the associations between environmental exposure and health outcomes. Therefore, the development and application of these -omics approaches to investigate the health impacts of environmental exposure are warranted.

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Li, Zhong-Min

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Publication list

Publications in peer-reviewed journals

1. **<u>Zhong-Min Li*</u>**, Florian Giesert, Daniela Vogt-Weisenhorn, Katharina Maria Main, Niels Erik Skakkebæk, Hannu Kiviranta, Jorma Toppari, Ulla Feldt-Rasmussen, Heqing Shen, Karl-Werner Schramm, Meri De Angelis. Determination of thyroid hormones in placenta using isotope-dilution liquid chromatography quadrupole time-of-flight mass spectrometry. *J Chromatogr A.* 2018, 1534: 85-92.

2. **<u>Zhong-Min Li*</u>**, David Hernandez-Moreno, Katharina Maria Main, Niels Erik Skakkebæk, Hannu Kiviranta, Jorma Toppari, Ulla Feldt-Rasmussen, Heqing Shen, Karl-Werner Schramm, Meri De Angelis. Association of *In Utero* Persistent Organic Pollutant Exposure with Placental Thyroid Hormones. *Endocrinology*. 2018, 159(10): 3473-3481.

3. **<u>Zhong-Min Li*</u>**, Michael Albrecht, Hermann Fromme, Karl-Werner Schramm, Meri De Angelis. Persistent Organic Pollutants in Human Breast Milk and Associations with Maternal Thyroid Homeostasis. *Environ Sci Technol* 2019, 54(2): 1111-1119.

4. **<u>Zhong-Min Li*</u>**, Bärbel Benker, Qibei Bao, Bernhard Henkelmann, Bernhard Michalke, Jan Pauluschke-Fröhlich, Krzysztof Flisikowski, Karl-Werner Schramm, Meri De Angelis. Placental Distribution Endogenous and Exogenous Substances: A Pilot Study Utilizing Cryo-sampled Specimen Off Delivery Room. (preparing)

5. **<u>Zhong-Min Li*</u>**, Manuel Miller, Angelika Scheideler, Sonja Charlotte Schriever, Paul Pfluger, Jens Mittag, Karl-Werner Schramm, Meri De Angelis. Determination of 3-Iodothyroamine in Mouse Blood and Tissues using Liquid Chromatography with Tandem Mass Spectrometry. (preparing)

International conference contributions and proceedings

1. **Zhong-Min Li***, Florian Giesert, Daniela Vogt-Weisenhorn, Katharina Maria Main, Niels Erik Skakkebæk, Hannu Kiviranta, Jorma Toppari, Ulla Feldt-Rasmussen, Heqing Shen, Karl-Werner Schramm, <u>Meri De Angelis</u>. "A validated LC-Q-TOF-MS method for quantitative analysis of thyroxine and metabolites in placenta". The 20$^{\text{th}}$ European Congress of Endocrinology. Barcelona, Spain, 2018.

2. **Zhong-Min Li**, David Hernandez-Moreno, Katharina Maria Main, Niels Erik Skakkebæk, Hannu Kiviranta, Jorma Toppari, Ulla Feldt-Rasmussen, Heqing Shen, Karl-Werner Schramm, <u>Meri De Angelis</u>*. "Association of placental thyroid hormone concentrations with congenital cryptorchidism". The 20th European Congress of Endocrinology. Barcelona, Spain, 2018.

3. **Zhong-Min Li***, Florian Giesert, Daniela Vogt-Weisenhorn, Katharina Maria Main, Niels Erik Skakkebæk, Hannu Kiviranta, Jorma Toppari, Ulla Feldt-Rasmussen, Heqing Shen, <u>Karl-Werner Schramm</u>, Meri De Angelis. "LC-Q-TOF-MS-analysis for quantification of thyroxine and metabolites in placenta". The 2nd German-French Conference on Diabetes Research. Berlin, Germany, 2018.

4. <u>**Zhong-Min Li***</u>, Yan Wu, Heqing Shen, Karl-Werner Schramm, Meri De Angelis. "Associations between Gestational Diabetes Mellitus and Placental Thyroid Hormones". The 6th Helmholtz Diabetes Conference. Munich, Germany, 2018.

5. <u>**Zhong-Min Li***</u>, Yan Wu, Heqing Shen, Karl-Werner Schramm, Meri De Angelis. "Associations between Gestational Diabetes Mellitus and Placental Thyroid Hormones". The 6th DZD Diabetes Research School. Berlin, Germany, 2018.

6. **Zhong-Min Li*,** Kathrin Wolf, Brenda W.C. Bongaerts, Alexandra Schneider, Annette Peters, Jürgen Wittsiepe, Karin Halina Greiser, Saskia Hartwig, Alexander Kluttig, Wolfgang Rathmann, Bernhard Henkelmann, Karl-Werner Schramm, <u>Meri De Angelis</u>. "Sample Pooling Strategy in T2D Exposome Wide Association Studies of POPs in Serum". 2019 Exposome Symposium. Brescia, Italy, 2019.

7. <u>Meri De Angelis</u>[*], **Zhong-Min Li**, Jan Pauluschke-Fröhlich, Andreas Fritsche, Bernhard Michalke, Bärbel Benker, Bernhard Henkelmann[a], Heqing Shen, Karl-Werner Schramm. "Human Placental Exposomics–Origin to Onset of Disease". 2019 Exposome Symposium. Brescia, Italy, 2019.

8. **Zhong-Min Li***, <u>Karl-Werner Schramm</u>, Meri De Angelis. "Determination of Thyroxine and Metabolites in Various Biomatrices Using LC-MS and LC-MS/MS". IIT-IC 2019-Second International Meeting of the Priority Program SPP1629. Berlin, Germany, 2019.

9. **Zhong-Min Li*,** Kathrin Wolf, Brenda W.C. Bongaerts, Alexandra Schneider, Annette Peters, Jürgen Wittsiepe, Karin Halina Greiser, Saskia Hartwig, Alexander Kluttig, Wolfgang Rathmann, Bernhard Henkelmann, <u>Karl-Werner Schramm</u>, Meri de Angelis. "Sample Pooling Strategy in T2D Exposome Wide Association Studies of POPs in Serum". The 39th International Symposium on Halogenated Persistent Organic Pollutants. Kyoto, Japan, 2019.

10. Meri De Angelis[*], **Zhong-Min Li**, Jan Pauluschke-Fröhlich, Andreas Fritsche, Bernhard Michalke, Bärbel Benker, Bernhard Henkelmann[a], Heqing Shen, <u>Karl-Werner Schramm</u>. "Placentome Exposomics targets origin to onset of disease". The 39th International Symposium on Halogenated Persistent Organic Pollutants. Kyoto, Japan, 2019.

11. <u>**Zhong-Min Li***</u>, David Hernandez-Moreno, Katharina Maria Main, Niels Erik Skakkebæk, Hannu Kiviranta, Jorma Toppari, Ulla Feldt-Rasmussen, Heqing Shen, Karl-Werner Schramm, Meri De Angelis. "Persistent Organic Pollutants and the Association with Thyroid Hormone Homeostasis in Placenta". The 31st annual conference of the International Society for Environmental Epidemiology (ISEE2019). Utrecht, the Netherlands, 2019.

12. <u>**Zhong-Min Li***</u>, Michael Albrecht, Hermann Fromme, Karl-Werner Schramm, Meri De Angelis. "Associations between Persistent Organic Pollutants and Thyroid Hormones in Human Breast Milk". The 11th Conference by Young Researchers for Young Researchers (Interact). Munich, Germany, 2020.

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