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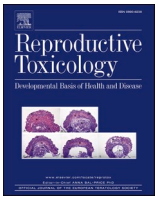

Xeno-estrogenic activity of real-life mixtures of perfluoroalkylated substances in human placenta homogenates

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ABSTRACT

Humans are simultaneously exposed to complex chemical mixtures, and its combined effect can affect human health. As part of the HBM4EU project, the actual mixture of perfluoroalkylated substances (PFAS) in 25 human placenta samples was extracted by chromatographic methods and assessed for xeno-estrogenic activity using two in-vitro bioassays: the estrogen receptor transactivity and the E-Screen assay. Most of the PFAS extracts displayed xeno-estrogenic activity, in one or both assays. The xeno-estrogenic activities in the two bioassays were not correlated, but both assays showed an overall negative correlation with placenta concentrations of single PFAS. Xeno-estrogenic activities were significantly related to maternal characteristics; being higher in young, smokers and primiparous women, but not with fetal growth (birth weight, birth length, head circumference, gestational age, placenta weight). The presented extraction method can be used to study the combined effect of real-life mixtures of PFAS in relation to health outcomes in large-scale human biomonitoring studies.

1. Introduction

Humans are simultaneously exposed to a large number of chemicals from various sources. The mixture exposure is raising concern for human health as chemicals may affect adverse health outcomes and can interact in an additive and/or synergistic way [1]. The effects of chemicals have mainly been studied with a one-chemical-at-a-time approach, and methods to assess the impact of chemical mixtures are still not fully developed and validated [2,3].

Suggested mathematical methods to assess human effects of chemical mixtures rely on characterizing the complex mixture composition and available toxicological information on all chemicals present in the mixture [4,5]. However, the methods are challenged by missing information on human exposure, toxicity, and possible unknown mixture

Abbreviations: AU, Aarhus University; BMI, body mass index; CV, coefficient of variation; DCCS-FBS, dextran-coated charcoal-stripped fetal bovine serum; DMEM, Dulbecco's Modified Eagle Medium; E2, 17β-estradiol; EFSA, European Food Safety Authority; ER, estrogen receptor; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; HBM4EU, the Human Biomonitoring for Europe initiative; PE, proliferative effect; PFAS, perfluoroalkylated substances; PFBA, perfluorobutanoic acid; PFBS, perfluorobutane sulfonate; PFDA, perfluorodecanoic acid; PFDoA, perfluorododecanoic acid; PFDS, perfluorodecane sulfonate; PFHpA, perfluoroheptanoic acid; PFHpS, perfluoroheptane sulfonate; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PFPeA, perfluoropentanoic acid; PFUnA, perfluoroundecanoic acid; PPAR, Peroxisome Proliferator-Activated Receptors; SPE, solid phase extraction; SRB, sulforhodamine B; UGR, University of Granada; WAX, weak anion exchange; XER, xeno-estrogenic receptor transactivities in non-competitive assay; XERcomp, xeno-estrogenic receptor transactivities in competitive assay.

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components, such as emerging chemicals [5,6]. Furthermore, synergistic or antagonistic interactions among the chemicals may be overlooked [1,7].

To overcome the challenges, new and integrative experimental approaches have been suggested to elucidate the link between real-life human chemical mixtures and health effects [8,9]. For example the extraction and isolation of mixtures from human biological samples and subsequent assessment of the combined/integrated effects in in-vitro bioassays [6]. Using this approach, we have previously linked in-vitro effects of real-life lipophilic chemical mixtures to health outcomes, including fetal growth [10,11], congenital malformations [12,13], early motor development in boys [14], sperm DNA damage [15,16], and breast cancer [17,18]. More recently, we have developed a method to extract real-life mixtures of perfluoroalkylated substances (PFAS) from human serum with simultaneously removing endogenous hormones [19]. After PFAS mixture extraction from serum of pregnant women, the xeno-estrogenic receptor transactivities (XER) of the PFAS mixtures were determined. We found that 61% of the 702 extracted PFAS mixtures agonized the estrogen receptor (ER) significantly and the serum PFAS-induced XER was inversely associated with birth weight and length [20,21].

The PFAS compounds are transferred from mother to fetus through the placenta [22]. Maternal serum PFAS levels during pregnancy is often used as an estimate of the fetal exposure in epidemiological studies. For instance, several studies have investigated the association between maternal serum PFAS levels at different times during pregnancy and fetal growth. The results are conflicting, but data mainly show inverse associations to fetal growth (reviewed in [23, 24]). Others have used cord blood PFAS levels with similar conflicting results [reviewed in 24]. Few studies have studied associations between placenta PFAS levels and birth weight. Hall et al. [25] found some sex-specific significant associations, while Bangma et al. [26] did not find any association in a high-risk pregnancy cohort.

In this context, the main objective of this study was to explore the possibilities of assessing the combined xeno-estrogenic activities of real-life PFAS mixtures in human placenta samples and associations with fetal growth. Specifically to apply the extraction method to 25 human placenta homogenates, and to determine the combined xeno-estrogenic effect of real-life PFAS mixture in two in-vitro estrogen-specific bioassays, the ER transactivity assay and the E-screen assay, and investigate possible associations with fetal growth.

2. Methods

2.4. Study population

As described previously [9], 25 placenta samples were selected from the biobank of San Cecilio University Hospital (Granada, Spain) from women participating in the INMA (INfancia y MedioAmbiente; Environment and Childhood) Granada birth cohort, collected between 2000 and 2002 [27]. Placentas were collected at time of delivery, weighed without fetal membranes/maternal decidua, and frozen at − 80 °C. The 25 placenta homogenates were randomly selected among those with a sufficient volume.

Maternal characteristics (age, body mass index (BMI), education, parity, smoking, and health/disease status) were obtained from medical records and validated questionnaires [13].

The INMA study followed the principles of the declaration of Helsinki and was approved by the Ethics Committee of San Cecilio University Hospital. All participating women signed the informed consent allowing the use of biological samples for environmental research purposes.

2.5. Placenta PFAS concentration

We measured 14 PFAS compounds in the placenta samples: perfluorobutane sulfonate (PFBS), perfluorohexane sulfonate (PFHxS), perfluoroheptane sulfonate (PFHpS), perfluorooctane sulfonate (PFOS), perfluorodecane sulfonate (PFDS), perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnA), and perfluorododecanoic acid (PFDoA). The PFAS concentrations were measured in the placenta tissue by combining salt-assisted liquid-liquid extraction with dispersive liquid-liquid micro-extraction and liquid chromatography-tandem mass spectrometry, following a previous validated protocol [28].

In addition to the single PFAS concentration, we calculated the sum of the four PFAS (PFHxS, PFOS, PFOA, and PFNA) included in the EFSA assessment, which contribute to approximately half of the total human exposure to PFAS [29].

2.6. Placenta PFAS extraction

We extracted the real-life PFAS mixtures with removal of endogenous hormones from 25 placenta homogenates using solid phase extraction (SPE), high-performance liquid chromatography (HPLC), and weak anion exchange (WAX) at the laboratory of Aarhus University (AU), Denmark. The extraction protocol was slightly modified from the previous published method of real-life PFAS mixtures in human serum samples [19].

The placenta homogenates (16 g per sample) were dissolved in hexane:water (1:1) and passed through a glass column with dried aluminium oxide 90 standardized (Merck KGaA, Darmstadt, Germany). The eluate was evaporated to 2–3 mL using a rotary evaporator, before SPE-HPLC-WAX extraction [19]. Briefly, the evaporated eluate were subjected to SPE (OASIS HLB, Waters) and liquid–liquid extraction (Hexane:EtOAc 9:1). The aqueous phase, containing PFAS, was further extracted with liquid–liquid extraction (tetrahydrofuran:hexane 3:2) and HPLC fractionation with a constant flow rate of 1.5 mL/min and two eluents: A) n-hexane, and B) n-hexane: isopropanol:methanol (40:15:45; vol:vol:vol). The elution gradient was 18 min: 10% B, 4 min: increase to 50% B (linearly), 13 min: decrease to 10% B (linearly), and 5 min: 10% B. The PFAS mixture fraction (F3) was collected between 22 and 26 min, and was further subject to WAX extraction (OASIS WAX, Waters) in which neutral compounds (e.g. E3 and E4) were eluted using 4 mL methanol and the PFAS mixture were eluted afterwards using 0.1% ammonium hydroxide in methanol. The placenta PFAS extracts were evaporated by vacuum centrifugation, and the dry fractions were stored at − 80 °C until determination of xeno-estrogenic activity [19]. In each extraction batch, a procedural blank consisting of hexane:water (1:1) were extracted in parallel.

Two independent SPE-HPLC-WAX extractions of real-life PFAS mixture were performed for each placenta homogenate. Two PFAS mixtures extracts from each placenta were used in two bioassays of ER-transactivity and E-screen. In each bioassay, one PFAS extract were tested in triplicates on the same plate.

2.7. Estrogen receptor (ER) transactivity assay

The ER-transactivity assay were conducted at Aarhus University (AU). The transactivation of ERs was evaluated with the stably transfected human breast adenocarcinoma MVLN cells carrying the estrogen response element luciferase reporter vector (provided by M. Pons, France). The procedure followed was previously described [30]. Briefly, cells were seeded at a density of 8.5×10^4 cells/well in 96-well plates (Perkin Elmer, Denmark) with culture medium that included phenol red-free Dulbecco's Modified Eagle Medium (DMEM) (LONZA, Belgium) supplemented with 1% dextran-coated charcoal-stripped fetal bovine serum (DCCS-FBS) (HyClone, USA), 6 μg/L insulin (Sigma, USA), 64 mg/L hexamycin (Sandoz, Denmark), 4 mM glutamine (Sigma, USA), and 20 mM HEPES (Gibco, UK), and were left to incubate at 37 °C with

5% CO_2 overnight. The experimental medium consisted of phenol red-free DMEM (LONZA, Belgium) containing 0.5% DCCS-FBS. The xeno-estrogenic activity of each placenta PFAS extract were tested alone (XER) and upon co-exposure with 24pM 17β-estradiol (E2; Sigma-Aldrich, Denmark) corresponding to approx. EC_{20} for competitive measurements (XERcomp). The dry extracts were reconstituted as previously described on the day of analysis [20], corresponding to 14.5 $g_{placenta}$/mL, and final test volume was 100 μL per well. Each extract was tested in a single assay with triplicates on the same plate. The cells were harvested after exposure to the placenta PFAS mixtures for 20 h, and cell luciferase activity and protein content measured [31]. A negative control (experimental medium), a solvent control (1.8% EtOH: DMSO: H2O (50:10:40)), and a positive control (EC_{20}: 25 pM E2) were included on each test plate and an E2 concentration–response control plate (1.5–300pM) was performed in parallel in each assay (Supp. Fig. S1). Furthermore, the antagonistic effect of ICI 182,780 (Fulvestrant) (Sigma-Aldrich, Denmark, >98% purity) was tested with a dose-response curve (5×10^{-11} – 5×10^{-5} M) in the presence of 50 pM E2 (Supp. Fig. S1).

The xeno-estrogenic receptor transactivities (XER) of the PFAS mixtures was expressed as percentage induction of the procedural blank control. In the competitive setup, the xeno-estrogenic receptor transactivation upon co-exposure with 24pM E2 (XERcomp) was expressed as percentage induction of the procedural blank control + 24pM E2. Samples with XER or XERcomp below or above 100% ± coefficient of variation (CV) was considered to display significant xeno-estrogenic activity (antagonistic or agonistic).

2.8. E-Screen assay

The E-Screen assay was conducted at University of Granada (UGR). MCF-7 cells were used to assess the cell proliferative effect (PE) of placenta extracts in the E-Screen assay, as described previously [9]. Briefly, cells were seeded at a density of 4×10^3 cells/well in 96-well plates (Falcon®, VWR International Eurolab, Spain) in culturing medium (DMEM with phenol red) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Spain), and incubated at 37 °C with 5% CO_2. After 24 h, culturing medium was replaced with experimental medium (phenol red-free DMEM) supplemented with 10% DCCS-FBS. Dried placenta extracts were reconstituted in 1 mL of experimental medium, filtered through a 0.22 μm filter, and tested at dilutions 1:1, 1:5 and 1:10, corresponding to 16 $g_{placenta}$/mL, 3.2 $g_{placenta}$/mL and 1.6 $g_{placenta}$/mL, respectively. The final test volume was 200 μL per well and each extract was tested in a single assay with triplicates on the same plate. In all experiments, a negative control (cells treated with hormone-free experimental medium), a solvent control (0.001% ethanol), and a positive control (100 pM E2, corresponding to approximately EC_{95}) were included on each assay plate, in addition with a parallel E2 dose-response plate (0.1–1000 pM) (Supp. Fig. S2). After 6 days of exposure, cells were fixed, stained with sulforhodamine B (SRB, Sigma-Aldrich, MO, USA) and the solubilized bound dye was read at 492 nm in a Titertek Multiscan plate reader (Flow, CA, USA). Furthermore, in Supp. Fig. S2 a dose-response curve of the antagonistic effect of ICI 182,780 (Fulvestrant) in the presence of 100 pM E2 is shown (Supp. Fig. S2).

The cell proliferation (PE) of the PFAS mixtures was expressed as the percentage proliferation of the negative control. Samples above the defined threshold of 150% were categorized as having significant positive PE.

2.9. Statistics

Statistical analyses were performed with IBM SPSS Statistics 28 and Microsoft Excel 2016. The statistical significance level was set at $p < 0.050$, and due to the low number of samples, $p < 0.100$ was considered as borderline significant.

Pearson correlations was used to assess the correlation between xeno-estrogenic activities (XER, XERcomp, and PE) and the concentrations of single PFAS in the placenta samples. Differences in xeno-estrogenic activities (XER, XERcomp, and PE) between maternal characteristic or fetal growth were tested with t-test (2 groups) or one-way ANOVA (>2 groups). ANCOVA was used for adjusted analyses with confounders previously shown to affect maternal PFAS concentrations and fetal growth [32,33], namely maternal age, educational level, parity, and smoking status. Associations between xeno-estrogenic activities and fetal growth were tested with multiple linear regression models, adjusting for the aforementioned covariates.

3. Results

3.4. Study population

In this study, the maternal median age and pre-pregnancy BMI were 31 years and 24.2 kg/m^2, respectively. The majority of women presented a normal weight (60%), while 40% were overweight or obese. Approximately half of the mothers (48%) received higher education (professional formation/university studies), 84% were non-smokers, and 52% were primiparous.

Of the 25 newborns, 44% were boys and 56% girls; and median gestational age was 40 weeks. Median birth weight, birth length, and head circumference were 3400 g, 51 cm, and 34 cm, respectively. The median placenta weight was 546 g. No infants were born preterm (<37 week) or with low birth weight (< 2500 g).

3.5. Xeno-estrogenic activity

The E2 dose-response curves are displayed in Fig. S1 and S2. Fig. 1 displays the xeno-estrogenic activities for the placenta PFAS-mixture extracts. In the ER transactivity assay, PFAS mixture extracted from the placentas were tested alone (XER) and in a competitive setup in the presence of the natural ER ligand, E2 (XERcomp) (Fig. 1A). When the extracts were tested alone, the median XER level was 114% of the control, and 76% (n = 19) of the samples were above the threshold value. In the competitive assay, the median XERcomp level was 124% of the control. In this case, 76% (n = 19) of the samples were above the upper threshold, 8% (n = 2) below the lower threshold value, and the remaining 16% (n = 4) within the threshold values (Fig. 1A).

In the E-Screen assay, the placenta PFAS extracts were tested at three concentrations, 1.6 $g_{placenta}$/mL, 3.2 $g_{placenta}$/mL, and 16 $g_{placenta}$/mL. In the lowest concentration, none of the extracts exceeded the threshold value, while 21% (n = 5) and 63% (n = 15) were above the threshold for the medium and highest concentrations, respectively (Fig. 1B). The median xeno-estrogenic PE was 104%, 109%, and 175% for the three tested concentrations, respectively. Subsequent statistical analyses used only the results obtained with the highest tested concentration (16 $g_{placenta}$/mL) for the best comparisons with the ER transactivity assay data using 14.5 g $_{placenta}$/mL.

No significant correlation was found between the xeno-estrogenic activities obtained in the two in-vitro bioassays (Table 1). Even though most sample activities were above the solvent control, the xeno-estrogenic activity of XERcomp and PE were weak to moderate negatively correlated with single PFAS concentrations in the placentas; specifically, XERcomp being significant with PFDA and borderline significant with PFHxS and PFOS, and for PE borderline significant with PFHxS, PFOS, and sum4PFAS (Table 1 and Fig. 2).

3.6. Xeno-estrogenic activity by maternal characteristics and fetal growth

The relations between xeno-estrogenic activities and maternal characteristics are shown in Table 2. The XERcomp differed significantly between the two age groups, with higher activity in younger women (≤30 years). Both XER (borderline significant) and PE (significant)

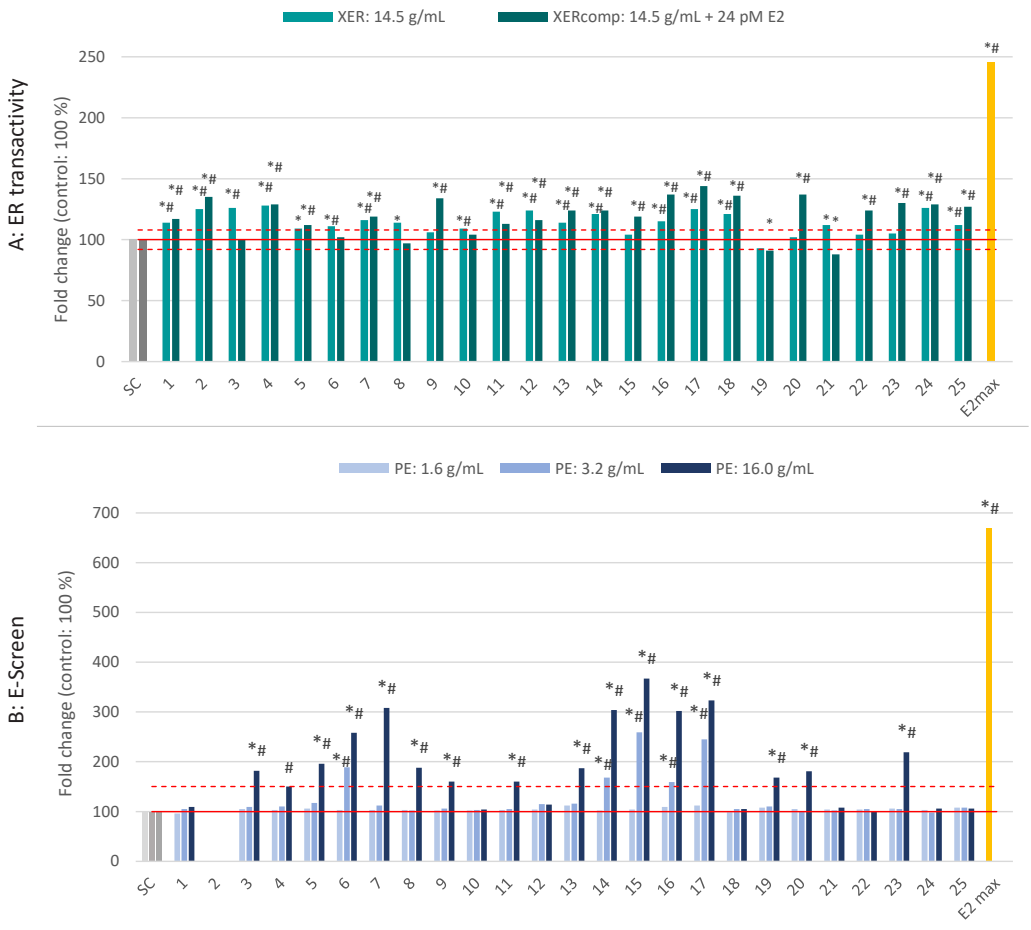

Fig. 1. Xeno-estrogenic activity of PFAS mixtures in placenta assessed with the estrogen receptor transactivity and the proliferative estrogenic bioassays. A: ER transactivation (reporter gene assay) of placental PFAS extracts tested at 14.5 g placenta/mL alone in non-competitive assay (XER: light green) or in the presence of EC20 = 24 pM E2 in competitive assay (XERcomp: dark green) in triplicates in a single independent experiment. The data is normalized to solvent controls (SC) set to 100 (grey bars and continuous red line). The E2 max (150 pM E2) is displayed by a yellow bar. Threshold values (100% ± CV) are indicated with dotted red lines, * highlight samples below or above the assay threshold value (100% ± CV), # highlight samples that are significantly different from the solvent control (t-test; $p < 0.05$). B: E-Screen assay measuring proliferation effect (PE) of placental PFAS extracts tested at 1.6 g placenta/mL (light blue), 3.2 g placenta/mL (medium blue), and 16.0 g placenta/mL (dark blue) in triplicates in a single independent experiment. The data is normalized to solvent controls (SC) set to 100 (grey bars and continuous red line). The E2 max (1 nM E2) is displayed in yellow bar. Threshold values are indicated with dotted red lines (>150%). * highlight samples below or above the assay threshold value (>150%), # highlight samples that are significantly different from controls (ANOVA test with post-hoc Dunnets test; $p < 0.05$). XER: Xeno-estrogenic receptor transactivation measured in the non-competitive assay ER reporter gene assay (without E2); XERcomp: Xeno-estrogenic receptor transactivation measured in the competitive assay ER reporter gene assay (with E2); PE: Xeno-estrogenic proliferative effect in the E-Screen assay .

Table 1
Pearson correlations between placenta PFAS-induced xeno-estrogenic activity and PFAS concentrations in placenta tissue.

	ER-transactivity assay				E-screen assay	
	XER (%)		XERcomp (%)		PE (%)	
	r_p	p	r_p	p	r_p	p
XER (%)			0.259	0.212	–0.029	0.893
XERcomp (%)	0.259	0.212			0.193	0.366
PE (%)	–0.029	0.893	0.193	0.366		
PFHxS (µg/kg)	0.002	0.994	–0.388	0.055#	–0.387	0.062#
PFOS (µg/kg)	0.052	0.803	–0.352	0.085#	–0.355	0.089#
PFOA (µg/kg)	0.110	0.601	–0.093	0.658	–0.261	0.218
PFNA (µg/kg)	0.047	0.824	–0.168	0.422	–0.210	0.324
PFDA (µg/kg)	0.090	0.668	–0.450	0.024 *	–0.167	0.434
sum4PFAS (µg/kg)	0.078	0.711	–0.299	0.147	–0.367	0.078#

XER: Xeno-estrogenic receptor transactivation measured in the non-competitive assay ER reporter gene assay (without E2); XERcomp: Xeno-estrogenic receptor transactivation measured in the competitive assay ER reporter gene assay (with E2); PE: Xeno-estrogenic proliferative effect in the E-Screen assay; PFHxS: perfluorohexane sulfonate; PFOS: perfluorooctane sulfonate; PFOA: perfluorooctanoic acid; PFNA: perfluorononanoic acid; PFDA: perfluorodecanoic acid: sum4PFAS: sum of PFHxS, PFOS, PFOA, PFNA. * Statistically significant ($p < 0.050$), # borderline statistically significant ($p < 0.100$).

xeno-estrogenic activity also differed by smoking habit, with higher activities among smokers. Primiparous women also showed higher PE activity than multiparous women (borderline significant). Placenta xeno-estrogenic activities (XER, XERcomp, and PE) did not differ according to BMI, educational level, or disease status (Table 2).

Placenta xeno-estrogenic activities in relation to fetal growth indices are shown in Table 3. Xeno-estrogenic activities did not differ by infant sex, birth weight, length, head circumference, gestational age, and placenta weight (Table 3). In linear regression analyses, xeno-estrogenic activities were not significantly associated with any of the fetal growth indices either (Table 4).

4. Discussion

In the present study, we explored the method use of extracting real-life PFAS mixture from placenta for further investigation of possible associations of PFAS induced xeno-estrogenic activities with fetal growth. The actual real-life PFAS mixture present in 25 human placenta samples was extracted with removal of endogenous hormones and assessed for xeno-estrogenic activity in two in-vitro bioassays, the ER transactivity assay and the E-Screen assay. When the extracted mixtures were tested alone (without E2), 40% of the samples showed significant xeno-estrogenic activity in both bioassays and, in addition, 52% had significant xeno-estrogenic activity in one of the assays (36% in the ER

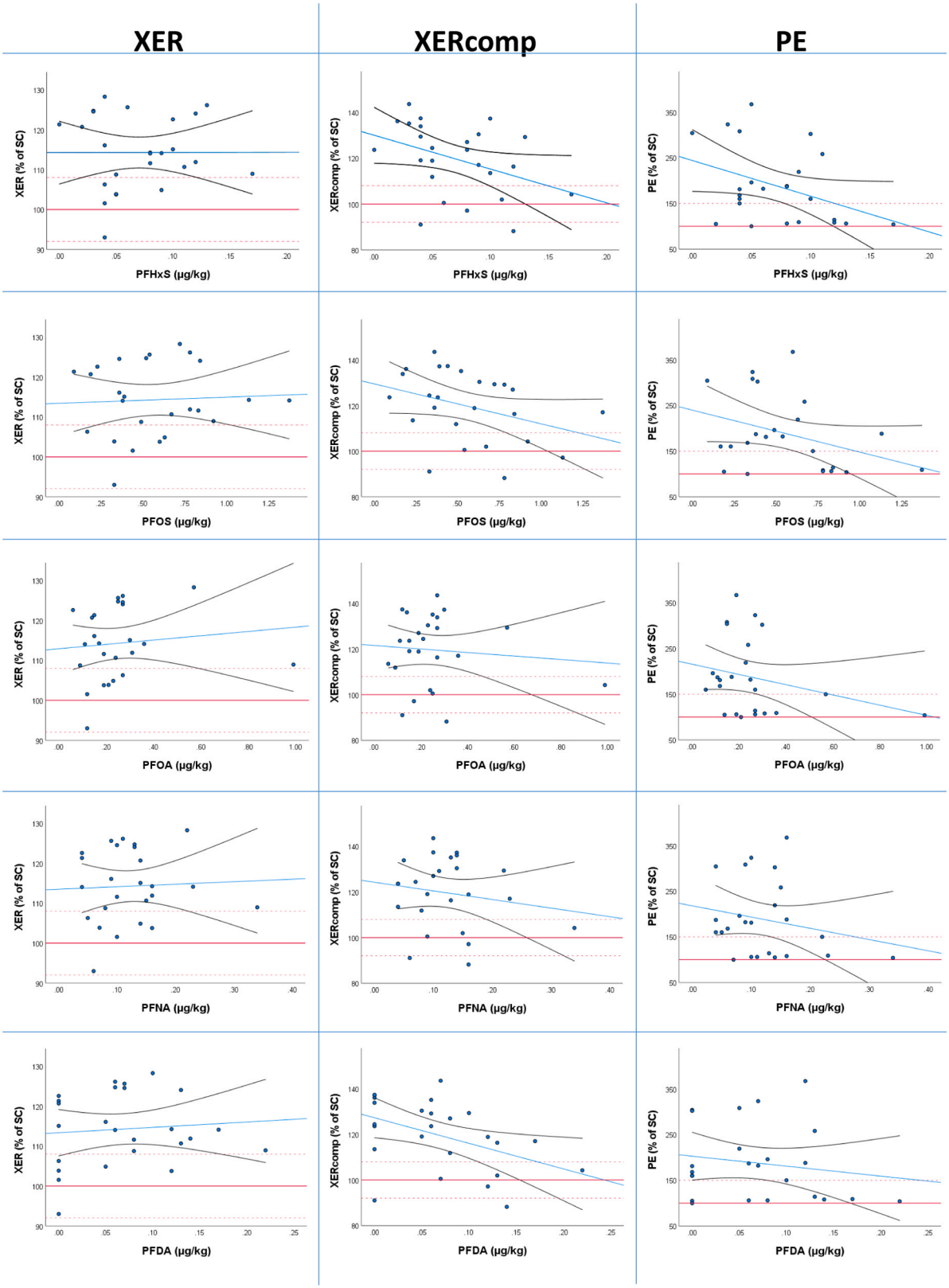

Fig. 2. Correlations of XER, XERcomp, and PE with the PFAS concentrations in placenta tissue. The graphs are displayed with PFAS placenta concentration on the x-axis and xeno-estrogenic activity on the y-axis. The xeno-estrogenic activity data is normalized to solvent controls (SC) set to 100 (solid red line), and threshold values (XER/XERcomp: 100% ± CV, PE >150) are indicated with dashed red lines. The correlations are displayed with blue lines with confidence intervals in black lines. XER: Xeno-estrogenic receptor transactivation measured in the non-competitive assay ER reporter gene assay (without E2); XERcomp: Xeno-estrogenic receptor transactivation measured in the competitive assay ER reporter gene assay (with E2); PE: Xeno-estrogenic proliferative effect in the E-Screen assay.

Table 2
Placenta PFAS-induced xeno-estrogenic activity (fold change) and maternal characteristics.

	n	ER transactivity assay				E-Screen assay	
		XER		XERcomp		PE	
		Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)
Total	25	114.3 (9.2)	114.1 (16.0)	119.6 (15.4)	123.6 (24.0)	187.7 (81.8)	174.5 (140.0)
Age (years)							
≤ 30	12	115.6 (7.7)	115.4 (15.0)	125.2 (8.2)	125.3 (15.0)	196.7 (98.4)	160.0 (197.0)
> 30	13	113.1 (10.5)	111.9 (18.0)	114.5 (18.7)	111.8 (33.0)	178.8 (64.2)	181.5 (76.0)
p-value[a]		*0.241*		*0.030* *		*0.414*	
BMI (kg/m^2)							
Normal weight (18.5–24.9)	15	115.9 (9.4)	116.1 (14.0)	118.1 (16.6)	119.1 (28.0)	186.1 (83.6)	175.0 (162.0)
Overweight & obese (≥25.0)	10	111.9 (8.7)	111.5 (13.0)	122.0 (13.7)	124.0 (24.0)	189.9 (83.6)	170.5 (78.0)
p-value[a]		*0.308*		*0.520*		*0.841*	
Educational level							
Primary	3	120.8 (6.0)	122.6 (NA)	112.5 (11.6)	113.5 (NA)	176.3 (14.4)	182.0 (NA)
Secondary	10	112.6 (11.6)	113.4 (19.0)	122.6 (15.5)	126.5 (21.0)	222.3 (89.3)	181.0 (151.0)
Professional Formation	7	115.1 (7.1)	111.9 (15.0)	110.6 (15.1)	111.8 (30.0)	131.7 (41.4)	108.0 (82.0)
University	5	112.5 (8.5)	114.1 (15.0)	130.5 (10.4)	130.4 (20.0)	210.6 (104.4)	219.0 (208.0)
p-value[b]		*0.575*		*0.123*		*0.131*	
Smoking							
No	21	112.8 (9.1)	111.9 (16.0)	117.9 (15.7)	118.9 (49.0)	173.2 (71.3)	164.0 (86.0)
Yes	4	122.0 (4.4)	122.9 (8.0)	128.8 (10.6)	126.4 (20.0)	260.3 (103.2)	306.0 (164.0)
p-value[a]		*0.066*[#]		*0.199*		*0.046* *	
Parity							
Primiparous	12	112.9 (10.1)	114.2 (17.0)	114.7 (14.5)	116.2 (22.0)	213.2 (78.3)	187.0 (136.0)
Multiparous	13	115.6 (8.4)	114.1 (16.0)	124.2 (15.2)	129.2 (18.0)	166.2 (81.3)	114.0 (133)
p-value[a]		*0.463*		*0.142*		*0.097*[#]	
Disease							
No	18	114.6 (9.3)	114.2 (16.0)	116.7 (16.5)	119.0 (29.0)	179.1 (85.6)	160.0 (117.0)
Yes[c]	7	113.4 (9.3)	114.1 (16.0)	126.7 (9.7)	129.4 (20.0)	208.7 (73.3)	196.0 (152.0)
p-value[a]		*0.792*		*0.146*		*0.319*	

a t-test on ln transformed variables, b ANOVA test on ln transformed variables, c Disease types: hypothyroidisms (gestational), diabetes (gestational), venous insufficiency, otosclerosis, idiopathic thrombocytopenic purpura. n = number of study participants in the group; XER: Xeno-estrogenic receptor transactivation measured in the non-competitive assay ER reporter gene assay (with E2); XERcomp: Xeno-estrogenic receptor transactivation measured in the competitive assay ER reporter gene assay (with E2); PE: Xeno-estrogenic proliferative effect in the E-Screen assay; SD: standard deviation; IQR: interquartile range; NA: not applicable; * statistically significant ($p < 0.050$), # borderline statistically significant ($p < 0.100$).

transactivity assay and 16% in the E-Screen assay), whereas only 2 samples (8%) showed no activity in the selected in-vitro bioassays. In the competitive ER transactivity assay (with E2; XERcomp), PFAS further-enhanced the E2 (EC_{20}) induced ER activity. Still with values being above the solvent controls (with E2), a significant negative correlation was observed between the PFAS mixture induced XERcomp and the single PFDA placenta concentration. Furthermore, both the XERcomp and E-Screen data demonstrated weak to moderate negative correlations with the single PFAS placenta concentrations in general, being borderline significant or non-significant. The xeno-estrogenic activities was also significantly related to some maternal characteristics (age, smoking, and parity), but no significant associations were found with fetal growth.

4.4. Xeno-estrogenic activity of PFAS compounds in the ER transactivity and E-Screen assays

Xeno-estrogenic activities induced by single PFAS have been reported in several studies. Using an ER transactivity assay, many studies have found that PFAS compounds can activated the ER alone and/or enhanced the E2-induced transactivity [31,34–40]. Few studies have reported antagonizing effects in the presence of E2 [37,40] or no effects [41]. PFOS and PFOA are the most studied compounds, but few other PFAS have also been studied, and variable xeno-estrogenic effects have been found [31,38–40]. Benninghoff et al. [38] found that PFOA, PFNA, PFDA, and PFOS activated ER in the non-competitive assay with PFOS being the most effective, whereas no effect was seen for PFUnA. Similarly, using the same ER transactivity assay as in the present study, we previously observed a stronger xeno-estrogenic effect of PFOS than for PFHxS and PFOA, while no effect was seen for PFNA, PFDA, PFUnA, and PFDoA analyzed as single compounds [31]. Behr et al. [39] also observed that PFOA and PFOS enhanced the E2 induced ERβ transactivity, while no effect was observed for shorter chained compounds (PFHxA, PFHxS, PFBA, and PFBS). Latest, Li et al. [40] reported increased ER transactivity for PFOS and PFHxS, decreased ER transactivity for PFBA, PFBS, and PFPeA, and no effect for PFHxA, PFHpA, PFOA, PFNA and PFDA in the non-competitive assay (without E2); while all these compounds antagonized the E2-induced activity at high concentrations in the competitive assay (with E2). We have also previously reported xeno-estrogenic activities and further increase of E2-induced activity for both an equimolar PFAS mixture and real-life serum extracted PFAS mixtures [18–21,31]. The xeno-estrogenic activity of single PFAS compounds has also been investigated using the E-Screen with conflicting results; however, to our knowledge, the E-Screen assay has not been used before to assess effect of PFAS mixtures. In the E-screen, some studies found that PFOS and PFOA elicited agonistic effects when tested alone and antagonistic effects in the presence of E2 [36,42]. However, Maras et al. [43] did not observe any xeno-estrogenic-induced cell proliferation of PFOS and PFOA, neither did Behr et al. [39] of several single PFAS (PFOA, PFOS, PFHxA, PFHxS, PFBA, and PFBS).

In the present study, we found xeno-estrogenic (agonistic and E2-enhancing) effects of the real-life PFAS placenta mixture in the ER transactivity and agonistic effects in the E-Screen assay. Even though no significant correlations were seen between the two assays, non-significant positive correlation were seen between the xeno-estrogenic activities of XERcomp and E-Screen-PE. Some discrepancy between the two assay has also been seen when single PFAS have been tested [39] and an explanation could be the differences between the assays. Even though they evaluate the same pathway, the experimental endpoint and

Table 3
Placenta PFAS-induced xeno-estrogenic activity (fold change) and fetal growth indices.

	n	ER transactivity assay				E-Screen assay	
		XER		XERcomp		PE	
		Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)
Total	25	114.3 (9.2)	114.1 (16.0)	119.6 (15.4)	123.6 (24.0)	187.7 (81.8)	174.5 (140.0)
Infant sex							
Male	11	111.3 (10.2)	109.0 (16.0)	119.7 (15.4)	123.6 (30.0)	191.4 (77.8)	177.5 (90.0)
Female	14	116.6 (7.8)	115.6 (12.0)	119.6 (15.9)	121.3 (21.0)	185.1 (87.3)	170.5 (195.0)
p-value[a]		*0.143*		*0.979*		*0.716*	
Adj p-value[b]		*0.255*		*0.503*		*0.320*	
Birth weight (g)							
Below median (<3400)	12	117.1 (8.4)	118.4 (12.0)	121.4 (15.6)	123.6 (20.0)	184.8 (82.9)	170.5 (167.0)
Above median (≥3400)	13	111.7 (9.4)	110.7 (14.0)	118.0 (15.6)	118.9 (29.0)	190.7 (84.2)	178.0 (140.0)
p-value[a]		*0.149*		*0.611*		*0.851*	
Adj p-value[b]		*0.575*		*0.492*		*0.524*	
Birth length (cm)							
Below median (<51)	12	116.6 (10.8)	121.0 (17.0)	120.9 (14.5)	123.6 (16.0)	193.3 (79.5)	175.0 (160.0)
Above median (≥51)	13	112.1 (7.1)	111.6 (7.0)	118.4 (16.6)	118.9 (31.0)	182.1 (87.2)	170.5 (136.0)
p-value[a]		*0.273*		*0.666*		*0.655*	
Adj p-value[b]		*0.742*		*0.951*		*0.977*	
Head circumference (cm)							
Below median (<35)	14	116.8 (10.0)	121.0 (14.0)	110.2 (16.7)	123.6 (24.0)	178.9 (81.8)	160.0 (139.0)
Above median (≥35)	11	111.1 (7.2)	110.7 (9.0)	119.3 (118.9)	118.9 (26.0)	198.2 (84.5)	188.0 (149.0)
p-value[a]		*0.146*		*0.981*		*0.539*	
Adj p-value[b]		*0.898*		*0.631*		*0.110*	
Gestational age (days)							
Below median (<280)	12	114.9 (11.4)	118.7 (20.0)	119.8 (15.4)	121.3 (26.0)	211.5 (95.0)	168.0 (158.0)
Above median (≥280)	13	113.7 (7.0)	114.1 (8.0)	119.5 (15.9)	123.6 (26.0)	167.6 (65.8)	181.0 (101.0)
p-value[a]		*0.814*		*0.954*		*0.237*	
Adj p-value[b]		*0.880*		*0.865*		*0.784*	
Placenta weight (g)							
Below median (<546)	12	115.0 (7.7)	114.7 (13.0)	117.8 (16.9)	118.0 (31.0)	205.1 (83.6)	185.0 (182.0)
Above median (≥546)	13	113.7 (10.6)	111.6 (19.0)	121.3 (14.2)	127.0 (22.0)	170.3 (79.6)	155.0 (105.0)
p-value[a]		*0.681*		*0.659*		*0.285*	
Adj p-value[b]		*0.469*		*0.744*		*0.675*	

a t-test on ln transformed variables, b: ANCOVA test adjusted for maternal age, parity, educational level, and smoking status; n = number of study participants in the group; XER: Xeno-estrogenic receptor transactivation measured in the non-competitive assay ER reporter gene assay (without E2); XERcomp: Xeno-estrogenic receptor transactivation measured in the competitive assay ER reporter gene assay (with E2); PE: Xeno-estrogenic proliferative effect in the E-Screen assay; SD: standard deviation; IQR: interquartile range.

Table 4
Association between placenta PFAS-induced xeno-estrogenic activities and fetal growth indices.

	ER transactivity assay				E-Screen assay	
	XER		XERcomp		PE	
	β (95% CI)	p	β (95% CI)	P	β (95% CI)	p
Birth weight (g)						
Unadjusted	-133.40 (−406.95; 140.15)	0.324	-71.73 (−319.96; 176.50)	0.556	5.98 (−278.75; 290.71)	0.966
Adjusted	60.36 (−243.27; 363.98)	0.682	-11.60 (−277.48; 254.28)	0.928	96.29 (−208.43; 401.02)	0.515
Birth length (cm)						
Unadjusted	-1.18 (−2.71; 0.34)	0.122	-0.82 (−2.22; 0.57)	0.236	0.21 (−1.44; 1.85)	0.797
Adjusted	-0.22 (−2.01; 1.57)	0.798	-0.30 (−1.85; 1.26)	0.693	0.60 (−1.18; 2.38)	0.487
Head circumference (cm)						
Unadjusted	-0.56 (−1.81; 0.70)	0.366	-0.23 (−1.37; 0.91)	0.675	-0.06 (−1.37; 1.25)	0.925
Adjusted	0.32 (−1.11; 1.75)	0.643	-0.08 (−1.33; 1.17)	0.893	0.25 (−1.18; 1.69)	0.717
Gestational age (days)						
Unadjusted	0.11 (−5.75; 5.96)	0.970	0.81 (−4.42; 6.03)	0.752	-2.87 (−8.73; 3.00)	0.322
Adjusted	1.08 (−5.99; 8.15)	0.753	1.51 (−4.63; 7.65)	0.612	-1.68 (−8.81; 5.45)	0.626
Placenta weight (g)						
Unadjusted	1.19 (−77.85; 80.23)	0.975	14.08 (−56.39; 84.55)	0.683	-43.55 (−121.75; 34.64)	0.260
Adjusted	-15.32 (−102.25; 71.62)	0.716	28.12 (−50.97; 107.21)	0.829	-9.00 (−95.58; 77.58)	0.830

Change per IQR (XER: 16, XERcomp: 24, E-Screen: 140); Adjusted for maternal age, educational level, parity, and smoking status; XER: Xeno-estrogenic receptor transactivation measured in the non-competitive assay ER reporter gene assay (without E2); XERcomp: Xeno-estrogenic receptor transactivation measured in the competitive assay ER reporter gene assay (with E2); PE: Xeno-estrogenic proliferative effect in the non-competitive E-Screen assay.

sensitivity differ. A direct interaction between the PFAS and the ER ligand-binding domain is investigated in the ER transactivity assay, whereas a cell proliferation stimulation by xeno-estrogenicity is assessed in the E-Screen assay [44] giving no information about the mechanisms behind [45]. In the E-Screen assay, the cell proliferation could be induced through non-ER-dependent mechanisms without binding to the ER [46]. Some findings could suggest that the xeno-estrogenic activity of PFAS may not be solely ER-mediated. In the E-screen assay, PFAS increased the proliferation significantly in the presence of the receptor inhibitor ICI [36]. In the ER transactivity assay, when PFAS were tested

in the presence of ICI, both Sonthithai et al. [35] and Xin et al. [36] found that ICI inhibited the PFAS induced xeno-estrogenic response to a level comparable to the control, but still being non-significantly higher. We have obtained similar results for PFHxS, PFOS and PFOA using our ER transactivity assay (unpublished), suggesting that the PFAS induced ER transactivity might not be solely through binding to the ER ligand binding site. Li et al. presented ER antagonist effect of single PFAS alone and a "further-inhibition effects" of PFAS in the presence of ICI if the PFAS concentration was higher than the ICI concentration [40]. PFAS is also known to interfere with the Peroxisome Proliferator-Activated Receptors (PPARs) [47] and an interesting cross-talk between the ERs and PPARs exist. The PPARs can bind to the estrogen-response-element and shown to be able to both activate and inhibit the estrogen responsive genes [48,49]. Thus, it is possible that some of the xeno-estrogenic activity observed in the present study is modified or mediated through the PPARs. To fully understand the mechanisms responsible for the xeno-estrogenic activity of PFAS, further studies are required for both the E-Screen and the ER transactivation.

4.5. Correlation between xeno-estrogenic activity of the placenta PFAS mixtures and single PFAS concentrations

When investigating the correlation between the placenta PFAS mixture-induced ER transactivity and the single PFAS concentrations, we found results similar to our previous Greenlandic study with serum PFAS extracts [18]. Although most xeno-estrogenic activities of serum extracts were above the solvent control added E2 (further-E2 enhancing), negative correlations were seen between single PFAS concentrations and XERcomp being strongest and significant for PFDA. In the present study, we observed borderline significant negative correlations between XERcomp and the single PFSA compounds (PFHxS and PFOS), while the negative correlations in the Greenlandic study were non-significant [18]. To understand the negative correlation between the PFAS-mixture induced xeno-estrogenic activity and the single PFAS placenta concentrations, it is important to remember that we did only measure the concentration of some of the PFAS expected to be present in the placenta extracts, and that only 5 of the 14 measured PFAS were above detection limit. The xeno-estrogenic activity of the PFAS extract is the combined effect of the mixture including the additive/synergistic interaction. Furthermore, when tested in the presence of E2, the PFAS extracts further enhanced the E2 response (n = 19, 78% of the samples), showing the agonistic potential of the PFAS mixture, even though the activity was negatively correlated with the placenta single PFAS concentrations. The exact mechanisms involved in the PFAS mixture modulation of the E2 response are not known but may involve the PPAR receptors as already discussed or PFAS may compete with the E2 response when present in high concentrations. In fact Li et al. suggested based on their studies that PFAS can attenuate endogenous ER signaling when E2 is co-administered, resulting in the disruption of estrogen-modulated endogenous gene expression in MCF-7 cells [40]. Our results is, however, in contrast to a Danish study, finding weak positive correlation between XERcomp and PFAS concentrations [20]. We are not able to fully explain the inconsistency between the studies, but it is most likely due to the different mixture compositions (further discussed below in 4.3). The XER was not significantly correlated with any of the single PFAS concentrations in neither this study nor any of the other studies, but non-significant weak positive tendencies were seen [18,20].

4.6. Association of real-life PFAS mixtures xeno-estrogenic activity and fetal growth

The xeno-estrogenic activity of the placenta PFAS mixture was not associated with fetal growth in the present study. This is in contrast to our previous Danish study, where the serum PFAS-mixture-induced XER were inversely associated with birth weight and length [20]. The Danish study included more than 700 pregnant women, and, thus, had a much higher statistical power than the present study (n = 25). This discrepancy could also be attributed to the difference in other factors, such as biological matrix and study population affecting the mixture composition. We have previously found different composition patterns between cohorts in Denmark, Norway, Greenland, and China, and even though the Danish and Norwegian composition were rather similar, the PFSAs tended to be highest in Norway and the PFCAs tended to be highest in Denmark [50]. Also when comparing the concentrations in the Danish cohort study [20,51] with previous Spanish studies [52,53], small differences in the mixture composition were seen, with higher PFOS and lower PFOA and PFHxS in the Danish study. Apart from the study population and mixture composition, the biological matrix also differs between the studies. The serum samples taken during early pregnancy were used in the Danish cohort study [20], whereas the PFAS mixture was extracted from placenta homogenates collected at time of delivery in the present study.

Only few studies have compared the PFAS concentration in maternal serum and placenta tissue [54–56], but the results indicate that the placenta/maternal serum ratio differs between PFAS compounds. Several factors affect the PFAS mixture composition, and thereby the xeno-estrogenic activity of the extracted PFAS mixture, including study population, geographic area, biological matrix, the trimester of sampling and fetal sex.

The most relevant biological matrix to study the association between PFAS exposure and fetal growth should be discussed. In addition to the biological matrix being available at the most relevant time of exposure, the target organ and mechanism also plays a critical role. PFAS can affect both molecular processes in the placenta and fetal tissues, although the mechanisms underlying the influence of PFAS on fetal growth are largely unknown. PFAS have been suggested to affect fetal growth through hormone disruption of the estrogen, androgen, and thyroid pathways, disruption of the PPAR pathway, changes in lipid metabolism, and oxidative stress [20,57–61]. Furthermore, PFAS could also affect placenta development and function, and as the placenta is critical for the nutrient and waste exchange, placenta insufficiency could negatively affect fetal growth and development [57,59,62]. Even though the placenta transfer efficiency for the PFAS differs according to carbon chain length and functional group [63], maternal PFAS levels still seem to be the best estimate of fetal exposure in early pregnancy, whereas umbilical cord blood and placenta tissue could be used to assess fetal exposure to PFAS and mixture composition in late pregnancy. In the present study, we were unfortunately not able to compare the xeno-estrogenic activities of serum and placenta PFAS extract mixtures from the same women.

PFAS concentrations found in placenta samples (median levels of PFHxS: 0.06 ng/g, PFOS: 0.52 ng/g, PFOA: 0.23 ng/g, PFNA: 0.11 ng/g) were lower than serum concentrations previously reported in the Spanish INMA birth cohort [52,53]. PFAS concentrations were 8–14 fold lower than those reported in maternal serum and 3–8 fold lower than those in umbilical cord serum [52,53]. Comparisons between the concentrations of these three biological matrices, placenta levels (ng/g) and maternal and cord serum levels (ng/mL), seem reasonable since the density of serum is approximately 1.024 g/mL [64]. PFAS mixtures were extracted from 16 g placenta, and different concentrations were used in the ER transactivity (14.5 $g_{placenta}$/mL) and E-Screen (1.6, 3.2, and 16.0 $g_{placenta}$/mL) assays. Thus, the placenta PFAS mixture extract analyzed in the in-vitro bioassays correspond well with those present in maternal serum, although somewhat elevated compared to cord serum levels.

The majority of the placenta PFAS extracts elicited xeno-estrogenic activity, and may potentially cause adverse health effects, even though we did not find a significant association with fetal growth. Estrogen regulates several important processes in the placenta, which may be disrupted by xeno-estrogenic compounds such as PFAS [65]. For instance, placenta estrogen has an important role in regulating the

placental angiogenesis, the initiation of labor, and the biosynthesis of progesterone; placental transfer mechanisms may modulate maturation of the fetal hypothalamic-pituitary-adrenocortical axis, and driving the changes in insulin sensitivity and glucose metabolism of the mother during pregnancy [65,66]. Studies have also suggested that boys may be more susceptible to the xeno-estrogenic effects of environmental chemicals than girls [11,14,67]. The effects of xeno-estrogenic compounds, like PFAS, on the placenta and fetus is of concern and must be further studied to elucidate mechanisms, sex differences, and potential adverse effects later in life.

4.7. Study limitations and strengths

This study was explorative and hypothesis-generating, and its strengths and limitations must be taken into account when drawing conclusions. A main strength is the extraction of the real-life mixture of placenta PFAS allowing us to take the interaction between the chemical compounds in the mixture into account and assessing the related effects. An additional strength is the application of two different in-vitro bioassays on the same set of human placentas, mapping different xeno-estrogenic signaling pathways and exploring their relationships.

One of the major limitations is the small number of placenta samples analyzed, limiting our ability to observe correlations between the two in-vitro bioassays, as well as associations between the observed xeno-estrogenic activities and fetal growth. Furthermore, due to the limited amount of biological sample, each extracted sample was only tested once (in triplicates = three wells on one plate) in each of the two xeno-estrogenic bioassays increasing the possibility of measurement errors. However, we believe that any measurement errors would be non-differential and not affecting the presented results. Even though the extracted PFAS mixture may provide a good estimate of the combined PFAS effects, the full chemical exposome was not addressed. When present in the human body, PFAS may interact with other exo- or endogenous chemicals. Furthermore, chemicals other than PFAS with similar chemical structure and chemical properties might be present in the extract, but the concentrations is expected to be very low since we have not seen any indication of other compounds than PFAS in the HPLC chromatograms [19]. In future studies, it would be desirable to explore the mixture composition with non-targeted chemical and additional fractionation methodologies, also making it possible to pinpoint the specific compounds generating the effect or comment on the interacting in the mixture. Another limitation of the study is the difference in methods used to extract the PFAS for single compound concentrations and the PFAS mixture for xeno-estrogenic activity. This may have affected the single PFAS recovery and concentrations in the two extracts. However, the significant correlation found for some of the compounds would counteract this limitation.

5. Summary & conclusion

In the present study, we employed a chromatographic extraction method to extract real-life PFAS mixtures from 25 human placenta homogenates. Most of the PFAS mixture extracts elicited xeno-estrogenic activities in two in-vitro estrogen-specific bioassays: the ER transactivity assay and the E-Screen cell proliferation assay. The PFAS-mixture related ER mediated xeno-estrogenic activities could further enhance the E2 induced activity and although above the solvent controls the PFAS mixture activities in both assays correlated negatively with increasing single PFAS concentrations measured in the placenta tissue. The xeno-estrogenic activities were significantly associated with some maternal characteristics; younger women (age less than 30 years), smokers and primiparous women showed higher xeno-estrogenic activity. However, the placenta PFAS mixture induced xeno-estrogenic activity was not significantly associated with fetal growth. The small sample number may explain some of the non-significant findings. This study had explorative and hypothesis-generating nature, and future larger studies are needed to fully elucidate the mechanisms and effect of placenta PFAS-mixture induced xeno-estrogenic activities on fetal growth.

Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.reprotox.2023.108444.

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