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1 **Association of prenatal perfluoroalkyl substance exposure and**  
2 **estrogen receptor 1 polymorphisms with the second-to-fourth**  
3 **digit ratio in school-aged children: the Hokkaido Study**

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16 2D:4D, ratio of the lengths of the second and fourth digits; AGD, anogenital distance; CI, confidence interval;  
17 CYP19, aromatase cytochrome P450; E<sub>2</sub>, estradiol; EDC, endocrine-disrupting chemical; ER, estrogen  
18 receptor; ESR1, estrogen receptor 1; MDL, method detection limit; OCC, Odense Child Cohort; PFAS,  
19 perfluoroalkyl substances; PFHpA, perfluoroheptanoic acid; PFHxA, perfluorohexanoic acid; PFHxS,  
20 perfluorohexane sulfonate; PFDA, perfluorodecanoic acid; PFDoDA, perfluorododecanoic acid; PFNA,  
21 perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PFTeDA,  
22 perfluorotetradecanoic acid; PFTrDA, perfluorotridecanoic acid; PFUnDA, perfluoroundecanoic acid; SNP,

1 single-nucleotide polymorphism; T<sub>2</sub>, testosterone; MEHP, mono(2-ethylhexyl) phthalate; DEHP, di(2-  
2 ethylhexyl) phthalate.

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1 **ABSTRACT**

2 Per- and Polyfluoroalkyl substances (PFAS) exert endocrine-disrupting  
3 effects. The ratio of the lengths of the second and fourth digits (2D:4D) is  
4 a noninvasive retrospective index of prenatal exposure to sex hormones,  
5 and estrogen receptor 1 (*ESR1*) polymorphisms may contribute to 2D:4D  
6 determination. We investigated whether *ESR1* polymorphisms modify the  
7 effects of prenatal PFAS exposure on 2D:4D. Participants ( $n=1,024$ ) with  
8 complete data in a prospective birth cohort study (the Hokkaido Study)  
9 were included, and PFAS concentrations were measured in maternal  
10 plasma collected in the third trimester. 2D:4D was determined from  
11 photocopies of palms of children using Vernier calipers. *ESR1*  
12 polymorphisms (rs2234693, rs9340799, and rs2077647) were genotyped  
13 by TaqMan polymerase chain reaction. The association of PFAS exposure  
14 and 2D:4D with *ESR1* polymorphisms was assessed by multiple linear  
15 regression adjusted for potential confounding factors. A 10-fold increase  
16 in maternal perfluorooctanoic acid (PFOA) concentration was associated  
17 with a 1.54% increase (95% confidence interval (CI): 0.40, 2.68) in mean  
18 2D:4D in children with the AA genotype at rs9340799 and a 2.24% increase

1 (95% CI: 0.57, 3.92) in mean 2D:4D in children with the AA genotype at  
2 rs2077647. A 10-fold increase in perfluorododecanoic acid (PFDoDA)  
3 concentration was associated with a significant increase in 2D:4D in  
4 children with the AA genotype [rs9340799, 1.18% (95% CI: 0.02, 2.34);  
5 rs2077647, 1.67% (95% CI: 0.05, 3.28)]. These associations were apparent  
6 among males. A significant gene-environment interaction between PFOA  
7 or PFDoDA exposure and *ESR1* polymorphism was detected. Thus, *ESR1*  
8 polymorphisms may modify the effects of prenatal PFAS exposure on sex  
9 differentiation.

10

11 **Keywords:** per- and polyfluoroalkyl substances, *ESR1* polymorphism,  
12 hand digit ratio, birth cohort, gene-environment interaction

1 **1. Introduction**

2 Per- and Polyfluoroalkyl substances (PFAS) have unique properties,  
3 including high stability and low surface tension, and have been widely  
4 used in industrial products, such as paper and textile coatings, polishes,  
5 food packaging materials, and fire-retarding foams, since the 1950s [1].  
6 The primary route of PFAS exposure is the consumption of contaminated  
7 food and drinking water, indoor air, and dust [2]. PFAS is commonly  
8 detected in human serum because of its long elimination half-life [2]. For  
9 example, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate  
10 (PFOS), the most widely used PFAS, have half-lives of 3.8 and 5.4 years,  
11 respectively [3]. Reportedly, PFAS exert endocrine-disrupting effects  
12 [4,5] and can cross the placenta [6]. Thus, prenatal exposure to PFAS  
13 may alter sex hormones *in utero* and affect the reproductive system [7,8];  
14 however, the adverse effects of PFAS on sex differentiation remain  
15 unclear.

16 The ratio of the lengths of the second and fourth digits (2D:4D) in  
17 human hands is considered to be determined by the 14<sup>th</sup> week of  
18 gestation, at which time it correlates negatively with prenatal androgen

1 levels and positively with prenatal estrogen levels [9-11]; therefore,  
2 2D:4D tends to be lower in males than in females [11]. Zheng and Cohn  
3 [12] reported that androgen receptors enhance chondrocyte proliferation  
4 in the fourth digit and are required for a low 2D:4D, whereas estrogen  
5 receptors (ERs) inhibit chondrocyte proliferation in the fourth digit and  
6 establish a relatively high 2D:4D. In humans, 2D:4D is used as a  
7 noninvasive retrospective index of prenatal exposure to sex hormones.

8         Previous epidemiological studies have extensively used the  
9 prenatal sexual dimorphic index of anogenital distance (AGD) to assess  
10 the reproductive toxicity associated with prenatal exposure to endocrine-  
11 disrupting chemicals (EDCs) [13,14]. However, animal experiments  
12 suggest that 2D:4D is more sensitive than AGD for assessing low-dose  
13 environmental exposure to EDCs [15]. Furthermore, 2D:4D is determined  
14 exclusively by prenatal exposure to sex hormones or compounds,  
15 whereas AGD is modulated by both prenatal and postnatal exposure  
16 [12,16]. Findings from these animal studies suggest that 2D:4D might be  
17 superior to AGD for evaluating the effects of prenatal EDC exposure on  
18 sex differentiation. To date, there have been three reports on the

1 association between human prenatal PFAS exposure and AGD [5,17,18];  
2 however, no study has described the association between prenatal PFAS  
3 exposure and human 2D:4D.

4 *In vivo* and *in vitro* experiments have demonstrated that PFAS can  
5 bind to ERs and exhibit environmental xenoestrogenic activity [19].

6 Moreover, animal studies have shown that PFAS exposure increases  
7 estradiol (E<sub>2</sub>) production and decreases testosterone (T<sub>2</sub>) production [20-  
8 22]. Furthermore, exposure to PFAS results in higher expression of the  
9 estrogen receptor 1 gene (*ESR1*) [20-22], encoding ER $\alpha$ , which plays a  
10 key role in both reproductive and nonreproductive tissues in the human  
11 body [23,24]. *ESR1* harbors two well-known, functional, single-nucleotide  
12 polymorphisms (SNPs) [*PvuII* (T>C, dbSNP: rs2234693) and *XbaI* (A>G,  
13 dbSNP: rs9340799)], along with the silent polymorphism rs2077647  
14 (A>G). These polymorphisms are associated with menarche, breast  
15 cancer, prostate cancer, and hypospadias [25-28]. We previously  
16 reported that *ESR1* polymorphisms are related to 2D:4D [29] and alter  
17 the association between prenatal exposure to di(2-ethylhexyl) phthalate  
18 (DEHP) and 2D:4D in 7-year-old children [30]. It is possible that *ESR1*

1 polymorphisms also affect the association between prenatal PFAS  
2 exposure and 2D:4D; however, no reports have described associations  
3 among prenatal PFAS exposure, *ESR1* polymorphisms, and sex  
4 differentiation. We aimed to evaluate the effects of prenatal PFAS  
5 exposure on sex differentiation in humans by examining the association  
6 between prenatal PFAS exposure and 2D:4D and to investigate whether  
7 the *ESR1* genotype alters the effects of prenatal PFAS on 2D:4D.

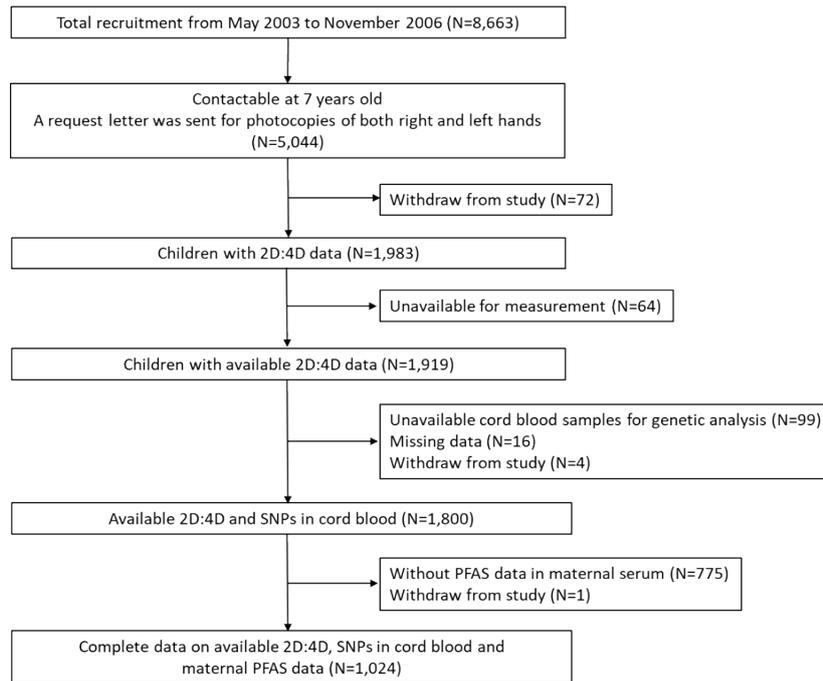
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## 9 **2. Material and methods**

### 10 *2.1. Participants and data collection*

11 This study was based on the Hokkaido large-scale cohort of the Hokkaido  
12 Study on Environment and Children's Health [31-33]. Briefly, 20,926  
13 local Japanese women who received routine prenatal healthcare in the  
14 first trimester (< 13 weeks of gestation) at 37 hospitals and clinics in  
15 Hokkaido prefecture were recruited from February 2003 to March 2012.  
16 A self-administered questionnaire during the first trimester of pregnancy  
17 was used to obtain baseline information, including maternal pre-  
18 pregnancy height, weight, parity, education level, household income,

1 alcohol consumption, and smoking habit. Birth records from hospitals  
2 provided the details of gestational age, infant sex, singleton or twin birth,  
3 and congenital anomalies. The criteria for the selection of participants  
4 included in this study have been described previously [29,30]. In all,  
5 8,663 babies born between May 2003 and November 2006 were enrolled  
6 in this study. Briefly, 5,044 children who were contactable at 7 years of  
7 age were requested to provide photocopies of both palms, and  
8 photocopies were received from 1,983 children. Eventually, 1,024  
9 children were included in the present 2D:4D analysis after eliminating  
10 children without available birth records, data on maternal serum PFAS  
11 levels, or sufficient cord blood samples for genotyping (Figure 1). Digit  
12 length was measured using steel Vernier calipers, and the details of  
13 measurement have been described previously [29,30]. 2D:4D was  
14 calculated by dividing the length of the second digit by that of the fourth  
15 digit. The mean 2D:4D of each participant was calculated using the  
16 formula  $[(\text{right 2D:4D} + \text{left 2D:4D})/2]*100$ , expressed as a percentage,  
17 and used for further analyses.



1

2 **Fig. 1.** Flow chart of participant selection from the Hokkaido Study.

3 2D:4D, ratio of the lengths of the second and fourth digits in human

4 hands; SNP, single-nucleotide polymorphism; PFAS, perfluoroalkyl

5 substances.

6

## 7 *2.2. PFAS concentration measurement*

8 Blood samples had been collected at 25 to 41 weeks of gestation

9 (median: 29 weeks of pregnancy) and stored at  $-80^{\circ}\text{C}$  until analysis. We

10 analyzed the concentration of 11 PFAS in maternal plasma samples,

11 namely perfluorohexane sulfonate (PFHxS), perfluorohexanoic acid

12 (PFHxA), perfluoroheptanoic acid (PFHpA), PFOS, PFOA,

1 perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA),  
2 perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid  
3 (PFDoDA), perfluorotridecanoic acid (PFTrDA), and  
4 perfluorotetradecanoic acid (PFTeDA). The details of maternal blood  
5 sample preparation and analysis were described previously [34,35].  
6 Ultra-performance liquid chromatography coupled with triple quadrupole  
7 tandem mass spectrometry was used for PFAS analysis. The method  
8 detection limits (MDLs) were 0.10 ng/mL for PFHxA, PFHpA, PFDA,  
9 PFDoDA, PFTrDA, and PFTeDA; 0.11 ng/mL for PFUnDA; 0.13 ng/mL for  
10 PFOA; 0.15 ng/mL for PFHxS; 0.30 ng/mL for PFOS; and 0.31 ng/mL for  
11 PFNA. Samples with PFAS levels below the detection limit of the assay  
12 were assigned a value that was half the detection limit. The MDL values  
13 or median concentration of the 11 PFAS are provided in Supplemental  
14 Table S1.

15

### 16 *2.3. ESR1 genotyping in children*

17 Umbilical cord blood samples had been collected at delivery, and 400 µg  
18 of the samples was used to extract genomic DNA. The genotyping of

1 rs2234693, rs9340799, and rs2077647 polymorphisms was performed  
2 using TaqMan polymerase chain reaction, according to manufacturer  
3 instructions (Applied Biosystems, Foster City, CA, USA). Detailed  
4 methods for the genotyping and assessment of genotyping quality have  
5 been described previously [29]. The dominant model comprised TT  
6 versus TC/CC for rs2234693, AA versus AG/GG for rs9340799, and AA  
7 versus AG/GG for rs2077647 [26,36]. Because the minor homozygous GG  
8 genotype frequency at rs9340799 (A>G) was low (3.2%; 33 children), we  
9 did not examine the recessive model in this study.

#### 10 *2.4. 2D:4D measurement*

11 Digit length was measured from ventral photocopies of both palms;  
12 details of the measurement process have been described previously  
13 [29,30]. The ratio was calculated by dividing the length of the second  
14 digit by that of the fourth digit. The mean 2D:4D value of each  
15 participant (calculated as  $[(\text{right } 2\text{D}:4\text{D} + \text{left } 2\text{D}:4\text{D})/2]*100$ ) was  
16 expressed as a percentage and used for further analyses.

17

#### 18 *2.5. Statistical analysis*

1 The data obtained from the children and mothers are expressed as means  
2 or percentages. The characteristics of the 1,024 children were compared  
3 with those of 1,919 children for whom 2D:4D data were obtained using  
4 one-sample *t*-tests. Data for three PFAS (PFHxA, PFHpA, and PFTeDA)  
5 were not examined by multiple linear regression because of the low  
6 detection rate (PFHxA, 35.2%; PFHpA, 32.3%; and PFTeDA, 8.5%). The  
7 median concentrations of PFAS and each genotype were compared using  
8 Mann-Whitney *U* tests. PFAS concentrations lesser than the MDLs were  
9 replaced with half of the MDL values. Because the PFAS concentrations  
10 in this study did not follow a normal distribution, we treated PFAS  
11 concentrations as continuous variables on a  $\log_{10}$  scale. The association  
12 of PFAS concentrations and 2D:4D was assessed using multiple linear  
13 regression adjusted for covariates, such as the sex and birth weight of  
14 the child and maternal age, parity, and alcohol consumption or smoking  
15 in the first trimester. We selected covariates based on previous reports  
16 that evaluated the predictors of 2D:4D [29,30]. Further, in addition to  
17 these covariates, we included maternal mono(2-ethylhexyl) phthalate  
18 (MEHP) concentration in the first trimester in the covariates to examine

1 the association between PFAS levels and 2D:4D independent from  
2 prenatal exposure to DEHP. MEHP, which is the primary and  
3 predominant metabolite of DEHP, is the only phthalate metabolite that  
4 showed feminizing effects on 2D:4D in our previous study [30].  
5 Additionally, we used multiple linear regression to evaluate the  
6 association of PFAS levels and *ESR1* polymorphisms with 2D:4D. The *p*-  
7 value for interaction ( $P_{\text{int}}$ ) was calculated using a post-estimation  
8 combined F-test for the two interaction variables between *ESR1*  
9 genotypes and PFAS. Moreover, we categorized PFAS concentrations as  
10 low (< 50<sup>th</sup> percentile) or high ( $\geq$  50<sup>th</sup> percentile) rather than as quartiles  
11 to avoid a reduction in the statistical power in each category after  
12 stratification by *ESR1* polymorphisms and sex. We examined 2D:4D in  
13 the high PFAS exposure group versus the low PFAS exposure group  
14 using multiple linear regression. The  $P_{\text{int}}$  for categorized analysis was  
15 defined as the PFAS level (assigned as 0 = low and 1 = high)  $\times$  child  
16 *ESR1* genotype (assigned as 0 = TT and 1 = TC/CC for rs2234693; 0 =  
17 AA and 1 = AG/GG for rs9340799; and 0 = AA and 1 = AG/GG for  
18 rs2077647). Statistical analyses were performed using the JMP Pro 14

1 software (SAS Institute, Cary, NC, USA). A two-sided  $p < 0.05$  was  
2 considered significant, and Bonferroni corrections were used for multiple  
3 comparisons.

4

#### 5 *2.6. Ethical considerations*

6 This study was approved by the Institutional Ethical Board for  
7 Epidemiological Studies at the Hokkaido University Graduate School of  
8 Medicine and Hokkaido University Center for Environmental and Health  
9 Sciences (latest cohort profile updated approved number: 136; approval  
10 date: September 3, 2021). The parents provided informed consent on  
11 behalf of the enrolled children. The Institutional Ethical Board for Human  
12 Gene and Genome Studies at Hokkaido University Graduate School of  
13 Medicine approved the study protocol.

14

### 15 **3. Results**

#### 16 *3.1. Cohort characteristics*

17 Participant characteristics are listed in Table 1. The participants included  
18 50.1% males and 49.9% females. Among the characteristics, only the

1 birth weight showed a significant difference with stratification by sex  
2 (males: 3,113 g versus females: 3,004 g;  $p < 0.001$ ). The major  
3 homozygous frequencies for rs2234693 (T>C), rs9340799 (A>G), and  
4 rs2077647 (A>G) were 31.9%, 65.2%, and 33.9%, respectively. The  
5 characteristics of the participants with complete data ( $n = 1,024$ ) and  
6 children with available 2D:4D data ( $n = 1,919$ ) are shown in  
7 Supplemental Table S2. In this study, the proportion of primiparous  
8 participants and maternal educational levels was greater than that in  
9 children for whom 2D:4D data were collected. We observed that the  
10 2D:4D values were significantly lower in boys than in girls (93.2% versus.  
11 94.6%, respectively;  $p < 0.001$ ). No child with congenital anomalies was  
12 included as a participant.

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6 **Table 1.** Participant characteristics ( $n = 1,024$ )

	<i>n</i> (%) or mean $\pm$ SD
<b>Maternal characteristics</b>	
Age at delivery (year)	31.1 $\pm$ 4.2
Pre-pregnancy body mass index (kg/m <sup>2</sup> )	20.9 $\pm$ 3.2
Parity	
Primiparous	477 (46.6)
Multiparous	542 (52.9)
Missing data	5 (0.5)
Annual household income (million yen per year)	
< 5	554 (54.1)
$\geq$ 5	353 (34.5)
Missing data	117 (11.4)
Education level (year)	
< 12	397 (38.8)
$\geq$ 13	620 (60.5)
Missing data	7 (0.7)
Smoking in the first trimester	
Nonsmoker	576 (56.3)
Smoker	228 (22.3)
Missing data	220 (21.5)
Alcohol consumption in the first trimester	
Nondrinker	841 (82.1)
Drinker	169 (16.5)
Missing data	14 (1.4)
<b>Children characteristics</b>	
Sex	
Male	513 (50.1)
Female	511 (49.9)
Birth weight (g)	3,059 $\pm$ 365
Gestational age (weeks)	38.8 $\pm$ 2.5
rs2234693(T>C)	

TT	327 (31.9)
TC	514 (50.2)
CC	183 (17.9)
TC/CC	697 (68.1)
rs9340799(A>G)	
AA	668 (65.2)
AG	323 (31.5)
GG	33 (3.2)
AG/GG	356 (34.8)
rs2077647(A>G)	
AA	347 (33.9)
AG	504 (49.2)
GG	173 (16.9)
AG/GG	677 (66.1)

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2

3 *3.2. PFAS concentration in maternal serum after stratification by ESR1*

4 *polymorphisms in children*

5 Supplemental Table S1 shows the distribution of the levels of 11 PFAS.

6 PFOS had the highest median concentration (6.06 ng/mL), followed by

7 PFOA (1.98 ng/mL), PFUnDA (1.36 ng/mL), PFNA (1.06 ng/mL), PFDA

8 (0.50 ng/mL), PFHxS (0.31 ng/mL), PFTrDA (0.33 ng/mL), and PFDoDA

9 (0.17 ng/mL). There were no significant differences in exposure levels

10 based on sex (data not shown). Supplemental Table S3 shows the median

11 (interquartile range) concentration of each PFAS stratified by *ESR1*

12 polymorphisms. There were no significant differences in the median

13 concentration of PFAS with respect to the *ESR1* genotype.

1

2 *3.3. Association between ESR1 polymorphisms and 2D:4D*

3 Supplemental Table S4 shows the associations between *ESR1*  
4 polymorphisms and 2D:4D. In dominant models, children with an *ESR1*  
5 polymorphism did not show a significant difference in the percentage  
6 change in 2D:4D compared with children in the reference group.

7 Furthermore, the results of the analysis indicated no association between  
8 *ESR1* polymorphisms and digit ratio.

9

10 *3.4. Association between PFAS levels and 2D:4D*

11 Table 2 shows the association between the (log<sub>10</sub>-transformed) PFAS  
12 concentration and 2D:4D. A 10-fold increase in the maternal  
13 concentration of PFAS was associated with a non-significant but  
14 increasing trend in 2D:4D in children, except for PFDA, which was  
15 associated with a non-significant but decreasing trend in 2D:4D.

16 Particularly, a 10-fold increase in the maternal PFOA concentration was  
17 associated with a 0.88% increase (95% CI: -0.02, 1.78) in 2D:4D in all  
18 participants; however, only males showed a statistically significant

1 association between PFOA and 2D:4D after stratification by sex (1.54%;  
 2 increase 95% CI: 0.33, 2.76).

3

4 **Table 2.** Association between PFAS concentrations and 2D:4D

	All <sup>a</sup>	Male <sup>b</sup>	Female <sup>b</sup>
	$\beta$ (95% CI)	$\beta$ (95% CI)	$\beta$ (95% CI)
PFHxS	0.40 (-0.34, 1.15)	0.57 (-0.47, 1.62)	0.16 (-0.94, 1.26)
PFOS	0.78 (-0.42, 1.97)	0.46 (-1.17, 2.09)	1.04 (-0.72, 2.79)
PFOA	0.88 (-0.02, 1.78)	1.54 (0.33, 2.76)*	0.20 (-1.15, 1.54)
PFNA	0.31 (-0.89, 1.50)	0.52 (-1.18, 2.22)	0.18 (-1.52, 1.88)
PFDA	-0.15 (-1.07, 0.77)	-0.17 (-1.37, 1.04)	-0.04 (-1.46, 1.39)
PFUnDA	0.33 (-0.20, 0.87)	0.44 (-0.26, 1.14)	0.21 (-0.60, 1.03)
PFDoDA	0.55 (-0.37, 1.47)	-0.10 (-1.40, 1.19)	1.12 (-0.20, 2.43)
PFTTrDA	0.44 (-0.26, 1.34)	0.66 (-0.59, 1.90)	0.21 (-1.12, 1.54)

5 <sup>a</sup> Multiple linear regression adjusted for sex, birth weight, maternal age, parity, alcohol consumption, and  
 6 smoking in the first trimester.

7 <sup>b</sup> Multiple linear regression adjusted for birth weight, maternal age, parity, alcohol consumption, and smoking  
 8 in the first trimester.

9 Because the PFAS levels were log<sub>10</sub>-transformed,  $\beta$  (95% CI) represents the expected percentage change in  
 10 2D:4D as a result of a 10-fold change in PFAS levels.

11 \*  $p < 0.05$ . 2D:4D, ratio of the lengths of the second and fourth digits; CI, confidence interval; PFAS,  
 12 perfluoroalkyl substances; PFHxS, perfluorohexane sulfonate; PFDA, perfluorodecanoic acid; PFDoDA,  
 13 perfluorododecanoic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS,  
 14 perfluorooctane sulfonate; PFTTrDA, perfluorotridecanoic acid; PFUnDA, perfluoroundecanoic acid.

15

16 *3.5. Effect of gene-environment interactions between ESR1*

17 *polymorphisms and PFAS on 2D:4D*

18 Gene-environment interactions between (log<sub>10</sub>-transformed) PFAS and

19 *ESR1* polymorphisms are shown in Table 3. With respect to the effect of

1 the interaction between PFOA and rs9340799 on 2D:4D, the expected  
2 change in 2D:4D per unit increase in the parameters considered was as  
3 follows: the PFOA level showed a mean increase of 0.58% (95% CI: -0.35,  
4 1.52), rs9340799-AA compared to AG/GG showed a mean decrease of  
5 0.01% (95% CI: -0.24, 0.22), and the interaction term between PFOA and  
6 rs9340799 showed a mean increase of 1.03% (95% CI: 0.15, 1.90), ( $P_{\text{int}} =$   
7 0.022). With respect to rs2077647, the expected change in 2D:4D per  
8 unit increase in the respective parameters considered was as follows: the  
9 PFOA level showed a mean increase of 1.24% (95% CI: 0.29, 2.19),  
10 rs2077647-AA compared to AG/GG showed a mean increase of 0.03%  
11 (95% CI: -0.20, 0.26), and the interaction term between PFOA and  
12 rs2077647 showed a mean increase of 1.06% (95% CI: 0.17, 1.95), ( $P_{\text{int}} =$   
13 0.020). The gene-environment interactions were apparent among males  
14 when the data were stratified by sex [rs9340799, 1.08% (95% CI: -0.10,  
15 2.25),  $P_{\text{int}} = 0.073$ ; rs2077647, 1.35% (95% CI: 0.12, 2.58),  $P_{\text{int}} = 0.032$ ].  
16 With respect to PFDODA, we detected marginal gene-environment  
17 interactions between rs9340799 and PFDODA on 2D:4D [all participants:  
18 0.87% (95% CI: -0.09, 1.83),  $P_{\text{int}} = 0.077$ , males: 1.31% (95% CI: -0.03,

1 2.65),  $P_{\text{int}} = 0.056$ , respectively]. In males, with respect to rs2077647, the  
2 expected changes in 2D:4D per unit increase in the respective  
3 parameters considered were as follows: PFDoDA showed a mean  
4 increase of 0.49% (95% CI: -0.90, 1.87), rs2077647-AA compared to  
5 AG/GG showed a mean decrease of 0.06% (95% CI: -0.38, 0.26), and the  
6 interaction term between PFOA and rs2077647 showed a mean increase  
7 of 1.65% (95% CI: 0.27, 3.03), ( $P_{\text{int}} = 0.019$ ). In addition, significant gene-  
8 environment interactions between PFNA and rs9340799 on 2D:4D was  
9 observed to be 1.55% (95%CI: 0.31, 2.79), ( $P_{\text{int}}=0.015$ ), as shown in  
10 Supplemental Table S5. The rs2234693 polymorphisms showed no  
11 interaction with PFOA or PFDoDA. Moreover, females did not show  
12 significant interactions between PFOA or PFDoDA and *ESR1*  
13 polymorphisms. Furthermore, no gene-environment interactions were  
14 observed between the remaining PFAS compounds and *ESR1*  
15 polymorphisms (Supplemental Table S5).

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6 **Table 3.** Effect of gene-environment interactions between PFAS and  
7 *ESR1* polymorphisms on 2D:4D

	All <sup>a</sup>	Male <sup>b</sup>	Female <sup>b</sup>
	$\beta$ (95% CI)	$\beta$ (95% CI)	$\beta$ (95% CI)
PFOA (ng/mL)	1.13 (0.16, 2.09)*	2.00 (0.59, 3.32)*	0.40 (-1.03, 1.83)
rs2234693-TT	0.01 (-0.23, 0.24)	-0.05 (-0.37, 0.27)	0.01 (-0.34, 0.36)
PFOA $\times$ rs2234693-TT	0.66 (-0.25, 1.56) $P_{int} = 0.157$	0.85 (-0.41, 2.12) $P_{int} = 0.186$	0.63 (-0.71, 1.97) $P_{int} = 0.359$
PFOA (ng/mL)	0.58 (-0.35, 1.52)	1.22 (-0.04, 2.49)	-0.07 (-1.47, 1.32)
rs9340799-AA	-0.01 (-0.24, 0.22)	0.03 (-0.28, 0.34)	-0.06 (-0.41, 0.29)
PFOA $\times$ rs9340799-AA	1.03 (0.15, 1.90)* $P_{int} = 0.022$	1.08 (-0.10, 2.25) $P_{int} = 0.073$	0.93 (-0.39, 2.26) $P_{int} = 0.167$
PFOA (ng/mL)	1.24 (0.29, 2.19)*	2.14 (0.81, 3.46)*	0.52 (-0.88, 1.91)
rs2077647-AA	0.03 (-0.20, 0.26)	-0.10 (-0.41, 0.22)	0.11 (-0.23, 0.46)
PFOA $\times$ rs2077647-AA	1.06 (0.17, 1.95)* $P_{int} = 0.020$	1.35 (0.12, 2.58)* $P_{int} = 0.032$	1.01 (-0.30, 2.33) $P_{int} = 0.130$
PFDODA (ng/mL)	0.64 (-0.34, 1.61)	0.40 (-1.03, 1.83)	0.97 (-0.39, 2.33)
rs2234693-TT	0.00 (-0.23, 0.24)	0.00 (-0.32, 0.32)	-0.01 (-0.35, 0.34)
PFDODA $\times$ rs2234693-TT	0.27 (-0.70, 1.24) $P_{int} = 0.588$	1.22 (-0.20, 2.64) $P_{int} = 0.093$	-0.55 (-1.92, 0.81) $P_{int} = 0.426$
PFDODA (ng/mL)	0.27 (-0.69, 1.24)	-0.45 (-1.80, 0.89)	0.96 (-0.45, 2.36)
rs9340799-AA	-0.01 (-0.25, 0.22)	0.01 (-0.30, 0.33)	-0.05 (-0.39, 0.30)
PFDODA $\times$ 9340799-AA	0.87 (-0.09, 1.83) $P_{int} = 0.077$	1.31 (-0.03, 2.65) $P_{int} = 0.056$	0.46 (-0.95, 1.86) $P_{int} = 0.522$
PFDODA (ng/mL)	0.78 (-0.18, 1.75)	0.49 (-0.90, 1.87)	1.14 (-0.21, 2.50)

rs2077647-AA	0.02 (-0.21, 0.26)	-0.06 (-0.38, 0.26)	0.10 (-0.24, 0.44)
PFDoDA × rs2077647-AA	0.78 (-0.18, 1.74)	1.65 (0.27, 3.03) *	0.05 (-1.31, 1.42)
	$P_{int} = 0.109$	$P_{int} = 0.019$	$P_{int} = 0.941$

<sup>a</sup> Multiple linear regression adjusted for sex, birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.

<sup>b</sup> Multiple linear regression adjusted for birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.

Because the PFAS levels were log<sub>10</sub>-transformed, β (95% CI) represents the expected percentage change in 2D:4D as a result of a 10-fold change in PFAS levels.

\*  $p < 0.05$ . 2D:4D, ratio of the lengths of the second and fourth digits; CI, confidence interval; PFAS, perfluoroalkyl substances; PFDoDA, perfluorododecanoic acid; PFOA, perfluorooctanoic acid.

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### 11 *3.6. Association between PFAS levels and ESR1 polymorphisms with* 12 *respect to 2D:4D*

13 Table 4 shows the association between the (log<sub>10</sub>-transformed) PFAS  
14 concentrations and 2D:4D stratified by *ESR1* polymorphisms. A 10-fold  
15 increase in the maternal PFOA concentration was associated with a  
16 1.54% increase (95% CI: 0.40, 2.68) in 2D:4D in children with the AA  
17 genotype of rs9340799, whereas children with the AG/GG genotype  
18 showed no significant change in 2D:4D. A 10-fold increase in PFOA  
19 concentration was associated with a 2.24% increase (95% CI: 0.57, 3.92)  
20 in 2D:4D in children with the AA genotype at rs2077647, whereas  
21 children with the AG/GG genotype showed no significant change in  
22 2D:4D. These associations were only apparent among males when the  
23 data were stratified by sex [rs9340799, 2.17% (95% CI: 0.66, 3.68);

1 rs2077647, 3.53% (95% CI: 1.15, 5.91)]. A 10-fold increase in the  
2 PFDoDA concentration was associated with a significant increase in  
3 2D:4D in children with the AA genotype at rs9340799 [a 1.18% (95% CI:  
4 0.02, 2.34) increase] or with the AA genotype at rs2077647 [a 1.67%  
5 (95% CI: 0.05, 3.28) increase], whereas no significant change in 2D:4D  
6 was observed in children with the AG/GG genotype. However, these  
7 associations were not significant after stratification by sex. Additionally,  
8 rs2234693 exerted no effect on the association between PFOA or  
9 PFDoDA concentrations and 2D:4D, and females showed no significant  
10 changes in the associations between PFOA or PFDoDA concentrations  
11 and 2D:4D after stratification by *ESR1* polymorphisms. Furthermore, no  
12 compound, besides PFOA or PFDoDA, was linked to 2D:4D after  
13 stratification by *ESR1* polymorphisms (Supplemental Table S6).

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18 **Table 4.** Association between PFAS and 2D:4D stratified by *ESR1*  
19 polymorphisms

Exposure	Genotype model	All <sup>a</sup>	Male <sup>b</sup>	Female <sup>b</sup>	
		$\beta$ (95% CI)	$\beta$ (95% CI)	$\beta$ (95% CI)	
PFOA	rs2234693	TT	1.26 (-0.43, 2.95)	2.02 (-0.39, 4.42)	0.64 (-1.76, 3.03)
		TC/CC	0.67 (-0.41, 1.75)	1.27 (-0.18, 2.71)	-0.05 (-1.70, 1.61)
	rs9340799	AA	1.54 (0.40, 2.68)*	2.17 (0.66, 3.68)*	0.88 (-0.87, 2.63)
		AG/GG	-0.35 (-1.82, 1.12)	0.39 (-1.72, 2.50)	-1.01 (-3.14, 1.13)
	rs2077647	AA	2.24 (0.57, 3.92)*	3.53 (1.15, 5.91)*	1.36 (-1.02, 3.74)
		AG/GG	0.24 (-0.84, 1.32)	0.73 (-0.71, 2.16)	-0.38 (-2.03, 1.27)
PFDoDA	rs2234693	TT	0.94 (-0.65, 2.54)	1.58 (-0.76, 3.92)	0.55 (-1.66, 2.77)
		TC/CC	0.33 (-0.80, 1.46)	-0.85 (-0.64, 2.17)	1.53 (-0.12, 3.17)
	rs9340799	AA	1.18 (0.02, 2.34)*	0.90 (-0.75, 2.55)	1.45 (-0.21, 3.11)
		AG/GG	-0.69 (-2.21, 0.83)	-1.80 (-3.96, 0.36)	0.33 (-1.89, 2.54)
	rs2077647	AA	1.67 (0.05, 3.28)*	2.17 (-0.19, 4.54)	1.35 (-0.94, 3.64)
		AG/GG	-0.03 (-1.15, 1.09)	-1.24 (-2.81, 0.33)	1.13 (-0.50, 2.77)

<sup>a</sup> Multiple linear regression adjusted for sex, birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.

<sup>b</sup> Multiple linear regression adjusted for birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.

Because the PFAS levels were log<sub>10</sub>-transformed,  $\beta$  (95% CI) represents the expected percentage change in 2D:4D as a result of a 10-fold change in PFAS levels.

\*  $p < 0.05$ . 2D:4D, ratio of the lengths of the second and fourth digits; CI, confidence interval; PFAS, perfluoroalkyl substances; PFDoDA, perfluorododecanoic acid; PFOA, perfluorooctanoic acid.

### 3.7. Association between PFAS levels (categorized as low or high) and

#### *ESR1* polymorphisms with regard to 2D:4D

Table 5 shows the associations between PFAS concentrations (low or high) and *ESR1* polymorphisms with regard to 2D:4D. Considering children with low exposure to PFOA and the AA genotype at rs9340799 as the reference group, children with high exposure to PFOA and the AA genotype had a 0.79% higher 2D:4D (95% CI: 0.23, 1.34), whereas

1 children with the AG/GG genotype showed no significant difference in the  
2 percent change in 2D:4D, regardless of PFOA exposure. Compared with  
3 children with low PFOA exposure and the AA genotype of rs2077647,  
4 children with high PFOA exposure and the AA genotype had a 1.19%  
5 higher 2D:4D (95% CI: 0.43, 1.96), whereas children with the AG/GG  
6 genotype showed no significant difference in the percent change in  
7 2D:4D. These associations were only apparent among males when the  
8 results were stratified by sex. Compared with children with low exposure  
9 to PFDoDA and the AA genotype, children with high exposure to PFDoDA  
10 and the AA genotype had a higher 2D:4D [rs9340799, 0.79% (95% CI:  
11 0.25, 1.33); rs2077647, 1.06% (95% CI: 0.31, 1.81)], whereas children  
12 with the AG/GG genotype showed no significant difference in the percent  
13 change in 2D:4D. These associations were apparent among males for  
14 rs2077647 when the results were stratified by sex. Notably, we found no  
15 significant effect of PFOA or PFDoDA exposure on 2D:4D with rs2234693  
16 polymorphisms and no significant associations between PFOA or PFDoDA  
17 exposure and *ESR1* polymorphism in 2D:4D among females. Moreover,

1 no other compounds were linked to 2D:4D or *ESR1* polymorphism (data  
 2 not shown).

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8 **Table 5.** Association between PFAS levels and *ESR1* polymorphisms with  
 9 regard to 2D:4D

Exposure	Genotype model	All <sup>a</sup>	Male <sup>b</sup>	Female <sup>b</sup>			
		$\beta$ (95% CI)	$\beta$ (95% CI)	$\beta$ (95% CI)			
PFOA	rs2234693	Low-TT	Reference	Reference	Reference		
		Low-TC/CC	0.28 (-0.37, 0.94)	0.49 (-0.44, 1.42)	0.23 (-0.71, 1.16)		
		High-TT	0.90 (0.11, 1.69)	1.35 (0.24, 2.46)	0.56 (-0.60, 1.71)		
		High-TC/CC	0.60 (-0.07, 1.27)	1.02 (0.04, 2.00)	0.27 (-0.67, 1.22)		
			rs9340799	Low-AA	Reference	Reference	Reference
				Low-AG/GG	0.44 (-0.22, 1.10)	0.36 (-0.53, 1.25)	0.48 (-0.17, 1.13)
		High-AA	0.79 (0.23, 1.34)*	1.03 (0.29, 1.77)*	0.49 (-0.01, 0.99)		
			High-AG/GG	0.36 (-0.30, 1.03)	0.54 (-0.38, 1.45)	0.18 (-1.21, 1.58)	
	rs2077647	Low-AA		Reference	Reference	Reference	
		Low-AG/GG	0.47 (-0.18, 1.11)	0.83 (-0.07, 1.72)	0.23 (-0.70, 1.16)		
		High-AA	1.19 (0.43, 1.96)*	1.71 (0.65, 2.78)*	0.81 (-0.29, 1.92)		
		High-AG/GG	0.60 (-0.06, 1.26)	1.16 (0.24, 2.09)*	0.12 (-0.83, 1.08)		
PFDoDA	rs2234693	Low-TT	Reference	Reference	Reference		
		Low-TC/CC	0.26 (-0.37, 0.88)	0.53 (-0.31, 1.37)	-0.07 (-0.99, 0.86)		
		High-TT	0.78 (0.01, 1.55)	1.26 (0.19, 2.33)	0.40 (-0.72, 1.52)		
		High-TC/CC	0.48 (-0.17, 1.12)	0.52 (-0.36, 1.41)	0.48 (-0.47, 1.43)		
	rs9340799	Low-AA	Reference	Reference	Reference		
		Low-AG/GG	0.50 (-0.13, 1.13)	0.49 (-0.36, 1.34)	0.54 (-0.39, 1.47)		

	High-AA	0.79 (0.25, 1.33)*	0.81 (0.08, 1.53)	0.82 (0.01, 1.64)
	High-AG/GG	0.20 (-0.95, 1.35)	0.01 (-0.88, 0.89)	0.43 (-0.58, 1.44)
rs2077647	Low-AA	Reference	Reference	Reference
	Low-AG/GG	0.38 (-0.24, 0.99)	0.77 (-0.06, 1.59)	0.03 (-0.89, 0.95)
	High-AA	1.06 (0.31, 1.81)*	1.41 (0.37, 2.46)*	0.84 (-0.26, 1.93)
	High-AG/GG	0.47 (-0.17, 1.11)	0.66 (-0.22, 1.54)	0.48 (-0.59, 1.28)

<sup>a</sup> Multiple linear regression adjusted for sex, birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.

<sup>b</sup> Multiple linear regression adjusted for birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.

$\beta$  (95% CI) represents the percentage change in 2D:4D, comparing with children with low exposure to PFAS and the *ESR1* genotype (AA genotype at rs9340799 and AA genotype at rs2077647) as the reference group.

\*  $p < 0.017$  (0.05/3) with Bonferroni correction. 2D:4D, ratio of the lengths of the second and fourth digits; CI, confidence interval; PFAS, perfluoroalkyl substances; PFDoDA, perfluorododecanoic acid; PFOA, perfluorooctanoic acid.

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### 11 *3.8. Association between PFAS and 2D:4D adjusted for (covariates* 12 *including) prenatal DEHP exposure*

13 Among the 1,024 children included in this study, we found 479 children  
14 with maternal MEHP data available from the first trimester. The median  
15 concentration of MEHP was 0.93 ng/mL, and there were no significant  
16 differences in MEHP levels according to sex (median MEHP; male: 0.83  
17 ng/mL, female: 1.00 ng/mL,  $p = 0.366$ ). Supplemental Table S7 shows the  
18 association between PFAS concentrations and 2D:4D; no statistical  
19 significance was observed. Supplemental Table S8 presents data for  
20 marginal gene-environment interaction between PFOA concentration and  
21 rs9340799 polymorphisms in males ( $P_{int} = 0.099$ ); however, no  
22 interactions were detected between PFDoDA and *ESR1* polymorphisms.  
23 As shown in Supplemental Table S9, a 10-fold increase in the maternal  
24 PFOA concentration was associated with a 3.21% increase (95% CI: 0.53,  
25 5.90) in 2D:4D in boys with an AA genotype at rs9340799 and a 4.52%

1 increase (95% CI: 0.05, 9.01) in boys with an AA genotype at rs2077647,  
2 whereas no association was observed between the PFDoDA  
3 concentration and 2D:4D stratified by *ESR1* polymorphisms.

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#### 5 **4. Discussion**

6 This is the first report describing associations between prenatal PFAS  
7 exposure *in utero* and 2D:4D in children with *ESR1* polymorphisms. We  
8 found that maternal PFOA and PFDoDA concentrations were associated  
9 with a feminizing effect on the 2D:4D in children with the AA genotype at  
10 rs9340799 or rs2077647. These findings suggest that PFAS may exhibit  
11 an endocrine-disrupting ability that adversely affects sex differentiation,  
12 even at relatively low-level exposure. Further, these effects were more  
13 apparent in males with specific *ESR1* polymorphisms.

14 In this study, the data demonstrated that PFAS exerts prenatal  
15 effects on 2D:4D. To date, three prospective birth cohort studies have  
16 investigated the association between prenatal PFAS exposure and AGD,  
17 but they have reported inconsistent findings [5,17,18]. The Odense Child  
18 Cohort (OCC) study of 3-month-old infants found that prenatal exposure  
19 to PFOS, PFHxS, PFNA, and PFDA was associated with a feminized AGD

1 in females, whereas no association was observed between PFAS exposure  
2 and AGD in males [5]. A prospective birth cohort study of male infants in  
3 China reported that maternal exposure to PFOS, PFDA, and PFUnDA was  
4 associated with feminized AGD at birth, whereas that to PFOS and  
5 PFTrDA was associated with feminized AGD at 6 months of age; however,  
6 these associations were not apparent at 12 months of age in the same  
7 infants [17]. In these two cohorts, even though the median maternal  
8 PFOA concentrations were comparable or considerably higher than those  
9 reported in the present study (present study: 1.98 ng/mL; OCC study:  
10 1.70 ng/mL; the Chinese study: 20.1 ng/mL), no statistically significant  
11 difference was detected in the association between PFOA and AGD. The  
12 effects of prenatal PFOA exposure on AGD were only detected in the  
13 Maternal-Infant Research on Environmental Chemicals cohort in Canada;  
14 however, maternal PFOA concentrations were correlated with a  
15 “masculinized” AGD in male newborns [18]. Although the median  
16 concentrations of PFOA in the present study were comparable to those in  
17 the Canadian cohort (1.71 ng/mL), the finding of the present study that  
18 PFOA exposure was associated with “feminized” 2D:4D is contradictory

1 to the finding reported in the Canadian study. The maternal PFDoDA  
2 concentration was only evaluated in the Chinese study, and was  
3 comparable to that in the present study (present study: 0.17 ng/mL; the  
4 Chinese study: 0.11 ng/mL) [17]. We identified the “feminizing” effects of  
5 PFDoDA on 2D:4D, whereas those of PFDoDA on AGD were detected only  
6 at higher PFDoDA concentrations (above the threshold value) [17]. The  
7 inconsistencies among the three AGD studies or between other AGD  
8 studies and the present study may have resulted from the differences  
9 between the outcome variables (2D:4D and AGD). First, 2D:4D may be  
10 superior to AGD as an index of sex differentiation owing to the sensitivity  
11 at low-dose exposure [15]. Second, 2D:4D is influenced by prenatal  
12 exposure, whereas AGD is modulated by postnatal exposure [12,16].  
13 Furthermore, the age at AGD measurement differed between each AGD  
14 study. Indeed, the study conducted in China [17] revealed that the  
15 prenatal effects of PFAS differed or disappeared based on the timing of  
16 AGD measurement, and the authors mentioned that AGD might be  
17 affected by postnatal PFAS exposure.

1 Previous *in vitro* studies have shown that PFOA exerts agonistic  
2 activity via ER $\alpha$ , through which it exerts its estrogenic activity [19],  
3 significantly induces ER transactivity [37], and promotes *ESR1*  
4 expression [20]. A study on rats demonstrated that PFOA exposure  
5 increased ER $\alpha$  expression [38], and cord blood PFOA levels were shown  
6 to be positively associated with E<sub>2</sub> levels in human fetuses [39]. These  
7 findings suggest that PFOA exposure might activate ER $\alpha$  and inhibit  
8 fourth digit development in children, eventually causing feminized  
9 2D:4D.

10 We found that the feminizing effects of PFAS on 2D:4D were only  
11 observed in male children. A possible explanation for these results is the  
12 action of the aromatase enzyme. Experiments in rats showed that  
13 androgen-stimulated aromatase expression or activity was higher in  
14 males than in females during the perinatal period [40,41]. In the human  
15 placenta, aromatase may convert prenatal androgen into prenatal  
16 estrogen [42]. Thus, a higher aromatase activity might increase the  
17 estrogen supply following conversion from androgens in male fetuses  
18 rather than in female fetuses. Additionally, the expression of the

1 aromatase cytochrome P450 gene (*CYP19*), which encodes aromatase,  
2 can increase in response to PFOA exposure. An *in vitro* study showed  
3 that PFOA exposure significantly induced CYP19 expression [20],  
4 increased E<sub>2</sub> production, and decreased T<sub>2</sub> production [20,43]. Aromatase  
5 activity was reportedly enhanced with PFOS exposure in human male  
6 infants [7]. Therefore, PFOA might enhance aromatase activity, and  
7 males with a higher PFOA exposure would have a higher estrogen supply  
8 than males with a lower exposure. Additionally, the evaluation of  
9 feminized effects in females is challenging, since a human female fetus  
10 develops under androgen deficiency [44]. In females, 2D:4D showed an  
11 increasing trend with respect to the association or interaction terms  
12 between PFOA and *ESR1* polymorphism. However, the effect sizes were  
13 smaller and non-significant in females, in contrast to the larger and  
14 statistically significant effect sizes in males. Therefore, the feminized  
15 effects on 2D:4D in females may be negligible.

16         The toxicokinetics of PFDoDA, which has been used as an  
17 alternative of PFOA or PFOS, are less studied. Some reports have shown  
18 that PFDoDA affects androgen or estrogen biosynthesis [45,46] and

1 reportedly acts as an ER agonist [19], or is involved in the dose-  
2 dependent increase in *ERS1* mRNA expression [47]. These reports  
3 suggest that PFDoDA, in addition to PFOA, might activate ER $\alpha$   
4 expression. With respect to its sex-dimorphic effects, a previous report  
5 has suggested potential PFDoDA-specific effects on CYP19 activity [47].  
6 The PFDoDA exposure-associated feminizing effects on males were less  
7 apparent than those attributed to PFOA in the present study. However,  
8 the PFDoDA level was lower than the PFOA level, implying that at levels  
9 as low as those observed in the present study, PFDoDA does not exert a  
10 significant influence on aromatase activity. The precise biological  
11 mechanism remains unclear, and further studies on the sex-dimorphic  
12 effects of PFDoDA are warranted.

13 In this study, we detected a significant gene-environment  
14 interaction between PFAS and *ESR1* polymorphism. Further, we  
15 observed a significant association between PFAS and 2D:4D in only the  
16 AA genotype at rs9340799 or rs2077647, suggesting that a specific allele  
17 in *ESR1* polymorphisms alters the effects of PFAS on 2D:4D. At  
18 rs9340799, the dominant A allele has been suggested to enhance ER $\alpha$

1 activity [26]; therefore, children with the AA genotype in rs9340799  
2 exposed to PFAS might show greater ER $\alpha$  activity. However, children  
3 with the AA genotype at rs2077647, which has been hypothesized to  
4 inhibit ER $\alpha$  activity [48], showed feminized 2D:4D with exposure to  
5 PFAS. This result was inconsistent with our expectation that children  
6 with the G allele would show feminized 2D:4D. Although the specific  
7 reasons underlying this inconsistency remain unclear, possibly,  
8 rs2077647 polymorphisms are in linkage disequilibrium with other  
9 polymorphisms that affect *ESR1* function or PFAS metabolism.

10 Additionally, there could be an affinity between PFAS and the A allele of  
11 rs2077647; however, additional studies are needed to confirm this, as  
12 there are no reports on the affinity between PFAS and specific *ESR1*  
13 polymorphisms. Although we observed a significant  $P_{int}$  between PFNA  
14 and rs9340799, we believe that this result was coincidental, because  
15 analysis between 2D:4D and PFNA exposure stratified by the rs9340799  
16 polymorphism did not reveal any significant differences.

17 Previously, we reported the associations of prenatal phthalate or  
18 BPA exposure with 2D:4D [30]. In detail, boys with the AG/GG genotype

1 at rs2077647 and higher DEHP exposure showed feminized 2D:4D.  
2 Phthalates and BPA are known to be estrogenic endocrine disruptors  
3 [49,50], and, in particular, DEHP has been suggested to enhance ER $\alpha$   
4 activity [51]. The precise mechanism by which DEHP activates ER $\alpha$  or  
5 *ESR1*, or the difference in the strength of estrogenic activity or in the  
6 affinity to *ESR1* polymorphisms between phthalates/BPA and PFAS,  
7 remains uncertain. Therefore, we cannot exclude the possibility of the  
8 interference of phthalates and BPA in the association between PFAS and  
9 2D:4D in this study. However, the expected change in 2D:4D per unit  
10 increase in PFOA concentration, considering adjustment for MEHP  
11 exposure, remained detectable to the same extent as that without  
12 adjustment for MEHP exposure. These results suggested that the  
13 estrogenic effects of PFOA on 2D:4D might be independent of the effects  
14 of DEHP. However, it should be noted that the sample size in analyses  
15 with MEHP reduced to less than half of the original sample size. Further,  
16 the smaller sample size reduced the statistical power, which may have  
17 caused the loss of statistical significance in the gene-environment  
18 interactions between PFOA or PFDoDA and *ESR1* polymorphisms, or in

1 the association between PFDoDA and 2D:4D stratified by *ESR1*  
2 polymorphisms.

3 The estrogenic activity of PFAS, or the mechanism by which it  
4 activates ER $\alpha$ , remains controversial. A number of *in vitro* mechanistic  
5 studies are available for PFAS; however, the results of these studies are  
6 inconsistent, even among studies reporting the absence of estrogenic  
7 activity of PFAS. Behr et al. [52] reported that PFAS did not affect ER  
8 activity at concentrations relevant to the human exposure level. Li et al.  
9 [53] demonstrated that the anti-estrogenic or estrogenic activities of  
10 PFAS may depend on the length of carbon chains, and PFAS might  
11 disturb the ER signaling pathway at environmentally relevant levels. The  
12 inconsistencies in the estrogenic activities of PFAS may be attributed to  
13 the use of different testing systems and the difference in the sensitivity  
14 among experiments or in estrogenic responses among species. Further  
15 studies are necessary to investigate the mechanism underlying the action  
16 of PFAS in ER $\alpha$  activity in humans.

17 The long-term effects of PFAS exposure *in utero* on human health,  
18 especially male reproduction, remains uncertain. Some cross-sectional

1 epidemiological studies have investigated whether PFAS exposure may  
2 exert adverse effects on adult male reproduction [54,55]. Only one study  
3 demonstrated the long-term adverse effects of PFAS exposure *in utero* on  
4 the adult male reproductive system in humans [8], in which prenatal  
5 exposure to PFOA was shown to potentially affect human adult male  
6 semen quality and reproductive hormone levels. Long-term follow-up is  
7 needed to clarify whether children with feminized 2D:4D exposed to  
8 PFAS in this study could develop clinical health problems in the future.

9         One of the strengths of this study was the prospective birth cohort  
10 design, which allowed us to estimate the effects of prenatal PFAS  
11 exposure on fetal sex differentiation. Additionally, we believe that our  
12 cohort of 1,024 children is the largest single cohort of its kind for  
13 evaluating the association between prenatal exposure to PFAS and sex  
14 differentiation in humans [5,17,18]. However, this study has some  
15 limitations. First, most maternal blood samples were collected during the  
16 third trimester, which is not the critical period for 2D:4D determination.  
17 However, owing to the long half-lives of PFAS, this inconsistency was  
18 expected to exert a low impact on the overall results. Second, we

1 categorized the exposure levels as high or low based on the sample size  
2 of the study. Analyses in four quartiles would have helped evaluate the  
3 PFAS dose-response effects more reliably, although this would require a  
4 larger study cohort than that available. Third, there was potential  
5 selection bias in this study. The study group had a higher proportion of  
6 primiparous participants and a higher maternal education level than the  
7 group from which 2D:4D data were obtained ( $n = 1,919$ ). Previous  
8 studies show that first pregnancy and higher maternal education levels  
9 are associated with higher maternal levels of PFAS [56,57]. Therefore, it  
10 is possible that the exposure levels in the study group were higher than  
11 that in the group from which 2D:4D data were obtained, and the effects  
12 of prenatal PFAS exposure on 2D:4D may have been overestimated.

13

## 14 **5. Conclusion**

15 In summary, we demonstrated that prenatal exposure to PFAS is  
16 associated with a higher (feminized) 2D:4D, and that children with the  
17 AA genotype at rs9340799 or rs2077647 showed a higher 2D:4D when  
18 exposed to PFOA or PFDoDA. These associations were apparent only

1 among males. These findings suggest that prenatal exposure to PFAS  
2 affects 2D:4D, and *ESR1* polymorphisms modify the effects of prenatal  
3 exposure to PFAS on 2D:4D.

4

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8

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17

## 18 **Conflicts of interest**

1 The authors declare that there are no conflicts of interest.

## 2 **References**

- 3 [1] A.M. Calafat, et al., Polyfluoroalkyl chemicals in the U.S. population: data from the  
4 National Health and Nutrition Examination Survey (NHANES) 2003–2004 and  
5 comparisons with NHANES 1999–2000, *Environ. Health Perspect.* 115 (2007)  
6 1596–1602. <https://doi.org/10.1289/ehp.10598>.
- 7 [2] A.B. Lindstrom, M.J. Strynar, E.L. Libelo, Polyfluorinated compounds: past, present,  
8 and future, *Environ. Sci. Technol.* 45 (2011) 7954–7961.  
9 <https://doi.org/10.1021/es2011622>.
- 10 [3] G.W. Olsen, et al., Half-life of serum elimination of  
11 perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in  
12 retired fluorochemical production workers, *Environ. Health Perspect.* 115 (2007)  
13 1298–1305. <https://doi.org/10.1289/ehp.10009>.
- 14 [4] C. Lau, et al., Perfluoroalkyl acids: a review of monitoring and toxicological findings,  
15 *Toxicol. Sci.* 99 (2007) 366–394. <https://doi.org/10.1093/toxsci/kfm128>.
- 16 [5] D.V. Lind, et al., Prenatal exposure to perfluoroalkyl substances and anogenital  
17 distance at 3 months of age in a Danish mother–child cohort, *Reprod. Toxicol.* 68  
18 (2017) 200–206. <https://doi.org/10.1016/j.reprotox.2016.08.019>.
- 19 [6] L.L. Needham, et al., Partition of environmental chemicals between maternal and  
20 fetal blood and tissues, *Environ. Sci. Technol.* 45 (2011) 1121–1126.  
21 <https://doi.org/10.1021/es1019614>.
- 22 [7] S. Itoh, et al., Association of perfluoroalkyl substances exposure *in utero* with  
23 reproductive hormone levels in cord blood in the Hokkaido Study on  
24 Environment and Children’s Health, *Environ. Int.* 94 (2016) 51–59.  
25 <https://doi.org/10.1016/j.envint.2016.05.011>.
- 26 [8] A. Vested, et al., Associations of *in utero* exposure to perfluorinated alkyl acids with  
27 human semen quality and reproductive hormones in adult men, *Environ. Health*  
28 *Perspect.* 121 (2013) 453–458. <https://doi.org/10.1289/ehp.1205118>.
- 29 [9] F. Galis, et al., Sexual dimorphism in the prenatal digit ratio (2D:4D), *Arch. Sex.*  
30 *Behav.* 39 (2010) 57–62. <https://doi.org/10.1007/s10508-009-9485-7>.
- 31 [10] M.A. Malas, et al., Fetal development of the hand, digits and digit ratio (2D:4D),  
32 *Early Hum. Dev.* 82 (2006) 469–475.  
33 <https://doi.org/10.1016/j.earlhumdev.2005.12.002>.

- 1 [11] J.T. Manning, et al., The ratio of 2nd to 4th digit length: a predictor of sperm  
2 numbers and concentrations of testosterone, luteinizing hormone and oestrogen,  
3 Hum. Reprod. 13 (1998) 3000–3004. <https://doi.org/10.1093/humrep/13.11.3000>.
- 4 [12] Z. Zheng, M.J. Cohn, Developmental basis of sexually dimorphic digit ratios, Proc.  
5 Natl Acad. Sci. U. S. A. 108 (2011) 16289–16294.  
6 <https://doi.org/10.1073/pnas.1108312108>.
- 7 [13] S.H. Swan, et al., First trimester phthalate exposure and anogenital distance in  
8 newborns, Hum. Reprod. 30 (2015) 963–972.  
9 <https://doi.org/10.1093/humrep/deu363>.
- 10 [14] E.S. Barrett, et al., First-trimester urinary bisphenol A concentration in relation to  
11 anogenital distance, an androgen-sensitive measure of reproductive  
12 development, in infant girls, Environ. Health Perspect. 125 (2017) 077008.  
13 <https://doi.org/10.1289/EHP875>.
- 14 [15] J. Auger, et al., Environmental levels of oestrogenic and antiandrogenic compounds  
15 feminize digit ratios in male rats and their unexposed male progeny, Proc. Biol.  
16 Sci. 280(1768) (2013) 20131532. <https://doi.org/10.1098/rspb.2013.1532>.
- 17 [16] D.J. Macleod, et al., Androgen action in the masculinization programming window  
18 and development of male reproductive organs, Int. J. Androl. 33 (2010) 279–287.  
19 <https://doi.org/10.1111/j.1365-2605.2009.01005.x>.
- 20 [17] Y. Tian, et al., Maternal plasma concentrations of perfluoroalkyl and polyfluoroalkyl  
21 substances during pregnancy and anogenital distance in male infants, Hum.  
22 Reprod. 34 (2019) 1356–1368. <https://doi.org/10.1093/humrep/dez058>.
- 23 [18] T.E. Arbuckle, et al., Prenatal perfluoroalkyl substances and newborn anogenital  
24 distance in a Canadian cohort, Reprod. Toxicol. 94 (2020) 31–39.  
25 <https://doi.org/10.1016/j.reprotox.2020.03.011>.
- 26 [19] A.D. Benninghoff, et al., Estrogen-like activity of perfluoroalkyl acids *in vivo* and  
27 interaction with human and rainbow trout estrogen receptors *in vitro*, Toxicol.  
28 Sci. 120 (2011) 42–58. <https://doi.org/10.1093/toxsci/kfq379>.
- 29 [20] G. Du, et al., Endocrine-related effects of perfluorooctanoic acid (PFOA) in  
30 zebrafish, H295R steroidogenesis and receptor reporter gene assays,  
31 Chemosphere. 91 (2013) 1099–1106.  
32 <https://doi.org/10.1016/j.chemosphere.2013.01.012>, H295R.
- 33 [21] G. Du, et al., Perfluorooctane sulfonate (PFOS) affects hormone receptor activity,  
34 steroidogenesis, and expression of endocrine-related genes *in vitro* and *in vivo*,  
35 Environ. Toxicol. Chem. 32 (2013) 353–360. <https://doi.org/10.1002/etc.2034>.

- 1 [22] J. Chen, et al., Chronic perfluorooctanesulphonic acid (PFOS) exposure produces  
2 estrogenic effects in zebrafish, *Environ. Pollut.* 218 (2016) 702–708.  
3 <https://doi.org/10.1016/j.envpol.2016.07.064>.
- 4 [23] J.F. Arnal, et al., Membrane and nuclear estrogen receptor alpha actions: from  
5 tissue specificity to medical implications, *Physiol. Rev.* 97 (2017) 1045–1087.  
6 <https://doi.org/10.1152/physrev.00024.2016>.
- 7 [24] M. Ponglikitmongkol, S. Green, P. Chambon, Genomic organization of the human  
8 oestrogen receptor gene, *EMBO J.* 7 (1988) 3385–3388.  
9 <https://doi.org/10.1002/j.1460-2075.1988.tb03211.x>.
- 10 [25] I. Stavrou, et al., Association of polymorphisms of the oestrogen receptor alpha  
11 gene with the age of menarche, *Hum. Reprod.* 17 (2002) 1101–1105.  
12 <https://doi.org/10.1093/humrep/17.4.1101>.
- 13 [26] S. Ban, et al., Genetic polymorphisms of ESR1 and ESR2 that may influence  
14 estrogen activity and the risk of hypospadias, *Hum. Reprod.* 23 (2008) 1466–  
15 1471. <https://doi.org/10.1093/humrep/den098>.
- 16 [27] M.R. Safarinejad et al., Estrogen receptors alpha (rs2234693 and rs9340799), and  
17 beta (rs4986938 and rs1256049) genes polymorphism in prostate cancer:  
18 evidence for association with risk and histopathological tumor characteristics in  
19 Iranian men, *Mol. Carcinog.* 51 Suppl 1 (2012) E104–E117.  
20 <https://doi.org/10.1002/mc.21870>.
- 21 [28] H. Johansson, et al., Impact of CYP19A1 and ESR1 variants on early-onset side  
22 effects during combined endocrine therapy in the TEXT trial, *Breast Cancer Res.*  
23 18 (2016) 110. <https://doi.org/10.1186/s13058-016-0771-8>.
- 24 [29] Y. Nishimura, et al., Association between ESR1 polymorphisms and second to  
25 fourth digit ratio in school-aged children in the Hokkaido Study, *Steroids.* 141  
26 (2019) 55–62. <https://doi.org/10.1016/j.steroids.2018.11.011>.
- 27 [30] Y. Nishimura, et al., Association of exposure to prenatal phthalate esters and  
28 bisphenol A and polymorphisms in the ESR1 gene with the second to fourth digit  
29 ratio in school-aged children: data from the Hokkaido study, *Steroids.* 159 (2020)  
30 108637. <https://doi.org/10.1016/j.steroids.2020.108637>.
- 31 [31] R. Kishi, et al., Cohort profile: the Hokkaido study on environment and children’s  
32 health in Japan, *Int. J. Epidemiol.* 40 (2011) 611–618.  
33 <https://doi.org/10.1093/ije/dyq071>.
- 34 [32] R. Kishi, et al., Ten years of progress in the Hokkaido birth cohort study on  
35 environment and children’s health: cohort profile--updated 2013, *Environ. Health*  
36 *Prev. Med.* 18 (2013) 429–450. <https://doi.org/10.1007/s12199-013-0357-3>.

- 1 [33] R. Kishi, et al., The Hokkaido Birth Cohort Study on Environment and Children's  
2 Health: cohort profile-updated 2017, *Environ. Health Prev. Med.* 22 (2017) 46.  
3 <https://doi.org/10.1186/s12199-017-0654-3>.
- 4 [34] E. Okada, et al., Temporal trends of perfluoroalkyl acids in plasma samples of  
5 pregnant women in Hokkaido, Japan, 2003–2011, *Environ. Int.* 60 (2013) 89–96.  
6 <https://doi.org/10.1016/j.envint.2013.07.013>.
- 7 [35] H. Goudarzi, et al., Prenatal exposure to perfluorinated chemicals and  
8 neurodevelopment in early infancy: the Hokkaido Study, *Sci. Total Environ.* 541  
9 (2016) 1002–1010. <https://doi.org/10.1016/j.scitotenv.2015.10.017>.
- 10 [36] J. Jurečková, et al., Estrogen receptor alpha polymorphisms and the risk of  
11 prostate cancer development, *J. Cancer Res. Clin. Oncol.* 141 (2015) 1963–1971.  
12 <https://doi.org/10.1007/s00432-015-1966-6>.
- 13 [37] L.S. Kjeldsen, E.C. Bonfeld-Jørgensen, Perfluorinated compounds affect the  
14 function of sex hormone receptors, *Environ. Sci. Pollut. Res. Int.* 20 (2013) 8031–  
15 8044. <https://doi.org/10.1007/s11356-013-1753-3>.
- 16 [38] Z. Qiu, et al., Binding specificities of estrogen receptor with perfluorinated  
17 compounds: A cross species comparison, *Environ. Int.* 134 (2020) 105284.  
18 <https://doi.org/10.1016/j.envint.2019.105284>.
- 19 [39] Q. Yao, et al., Cord blood Per- and polyfluoroalkyl substances, placental  
20 steroidogenic enzyme, and cord blood reproductive hormone, *Environ. Int.* 129  
21 (2019) 573–582. <https://doi.org/10.1016/j.envint.2019.03.047>.
- 22 [40] A.J. Andrade, et al., A dose–response study following *in utero* and lactational  
23 exposure to *di*-(2-ethylhexyl)-phthalate (DEHP): non-monotonic dose–response  
24 and low dose effects on rat brain aromatase activity, *Toxicology.* 227 (2006) 185–  
25 192. <https://doi.org/10.1016/j.tox.2006.07.022>.
- 26 [41] C.E. Roselli, et al., Sex differences in androgen-regulated cytochrome P450  
27 aromatase mRNA in the rat brain, *Endocrine.* 5 (1996) 59–65.  
28 <https://doi.org/10.1007/BF02738657>.
- 29 [42] Y. Li, et al., Expression of 3beta-hydroxysteroid dehydrogenase type 1, P450  
30 aromatase, and 17beta-hydroxysteroid dehydrogenase types 1, 2, 5 and 7 mRNAs  
31 in human early and mid-gestation placentas, *Placenta.* 26 (2005) 387–392.  
32 <https://doi.org/10.1016/j.placenta.2004.07.008>.
- 33 [43] J.S. Kang, J.S. Choi, J.W. Park, Transcriptional changes in steroidogenesis by  
34 perfluoroalkyl acids (PFOA and PFOS) regulate the synthesis of sex hormones in  
35 H295R cells, *Chemosphere.* 155 (2016) 436–443.  
36 <https://doi.org/10.1016/j.chemosphere.2016.04.070>.

- 1 [44] M. Welsh, et al., Identification in rats of a programming window for reproductive  
2 tract masculinization, disruption of which leads to hypospadias and  
3 cryptorchidism, *J. Clin. Invest.* 118 (2008) 1479–1490.  
4 <https://doi.org/10.1172/JCI34241>.
- 5 [45] Z. Shi, et al., Chronic exposure to perfluorododecanoic acid disrupts testicular  
6 steroidogenesis and the expression of related genes in male rats, *Toxicol. Lett.*  
7 188 (2009) 192–200. <https://doi.org/10.1016/j.toxlet.2009.04.014>.
- 8 [46] Z. Shi, et al., The effect of perfluorododecanonic acid on endocrine status, sex  
9 hormones and expression of steroidogenic genes in pubertal female rats, *Reprod.*  
10 *Toxicol.* 27 (2009) 352–359. <https://doi.org/10.1016/j.reprotox.2009.02.008>.
- 11 [47] O.R. Ibor, et al., Contaminant levels and endocrine disruptive effects in *Clarias*  
12 *gariepinus* exposed to simulated leachate from a solid waste dumpsite in  
13 Calabar, Nigeria, *Aquat. Toxicol.* 219 (2020) 105375.  
14 <https://doi.org/10.1016/j.aquatox.2019.105375>.
- 15 [48] S. Sathyanarayana, et al., A pilot study of the association between genetic  
16 polymorphisms involved in estrogen signaling and infant male genital  
17 phenotypes, *Asian J. Androl.* 14 (2012) 766–772.  
18 <https://doi.org/10.1038/aja.2012.27>.
- 19 [49] S. Rehman, et al., Endocrine disrupting chemicals and impact on male reproductive  
20 health, *Transl. Androl. Urol.* 7 (2018) 490–503.  
21 <https://doi.org/10.21037/tau.2018.05.17>.
- 22 [50] C.A. Harris, et al., The estrogenic activity of phthalate esters *in vitro*, *Environ.*  
23 *Health Perspect.* 105 (1997) 802–811. <https://doi.org/10.1289/ehp.97105802>.
- 24 [51] S. Takeuchi, et al., Differential effects of phthalate esters on transcriptional  
25 activities via human estrogen receptors alpha and beta, and androgen receptor,  
26 *Toxicology.* 210 (2005) 223–233. <https://doi.org/10.1016/j.tox.2005.02.002>.
- 27 [52] A.C. Behr, et al., Perfluoroalkylated substances (PFAS) affect neither estrogen and  
28 androgen receptor activity nor steroidogenesis in human cells *in vitro*, *Toxicol.*  
29 *Lett.* 291 (2018) 51–60. <https://doi.org/10.1016/j.toxlet.2018.03.029>.
- 30 [53] J. Li, et al., Evaluation of the estrogenic/antiestrogenic activities of perfluoroalkyl  
31 substances and their interactions with the human estrogen receptor by  
32 combining In Vitro Assays and In Silico Modeling, *Environ. Sci. Technol.* 54  
33 (2020) 14514–14524. <https://doi.org/10.1021/acs.est.0c03468>.
- 34 [54] U.N. Joensen, et al., PFOS (perfluorooctanesulfonate) in serum is negatively  
35 associated with testosterone levels, but not with semen quality, in healthy men,  
36 *Hum. Reprod.* 28 (2013) 599–608. <https://doi.org/10.1093/humrep/des425>.

- 1 [55] G. Toft, et al., Exposure to perfluorinated compounds and human semen quality in  
2 Arctic and European populations, *Hum. Reprod.* 27 (2012) 2532-2540.  
3 <https://doi.org/10.1093/humrep/des185>.
- 4 [56] A.L. Brantsæter, et al., Determinants of plasma concentrations of perfluoroalkyl  
5 substances in pregnant Norwegian women, *Environ. Int.* 54 (2013) 74-84.  
6 <https://doi.org/10.1016/j.envint.2012.12.014>.
- 7 [57] M.S. Tsai, et al., Determinants and temporal trends of perfluoroalkyl substances in  
8 pregnant women: the Hokkaido study on environment and children's health, *Int.*  
9 *J. Environ. Res. Public Health.* 15 (2018) .  
10 <https://doi.org/10.3390/ijerph15050989>.
- 11
- 12
- 13
- 14
- 15 [1] A.M. Calafat, et al., Polyfluoroalkyl chemicals in the U.S. population: data from the  
16 National Health and Nutrition Examination Survey (NHANES) 2003-2004 and  
17 comparisons with NHANES 1999-2000, *Environ. Health Perspect.* 115 (2007)  
18 1596-1602. <https://doi.org/10.1289/ehp.10598>.
- 19 [2] A.B. Lindstrom, M.J. Strynar, E.L. Libelo, Polyfluorinated compounds: past, present,  
20 and future, *Environ. Sci. Technol.* 45 (2011) 7954-7961.  
21 <https://doi.org/10.1021/es2011622>.
- 22 [3] G.W. Olsen, et al., Half-life of serum elimination of  
23 perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in  
24 retired fluorochemical production workers, *Environ. Health Perspect.* 115 (2007)  
25 1298-1305. <https://doi.org/10.1289/ehp.10009>.
- 26 [4] C. Lau, et al., Perfluoroalkyl acids: a review of monitoring and toxicological findings,  
27 *Toxicol. Sci.* 99 (2007) 366-394. <https://doi.org/10.1093/toxsci/kfm128>.
- 28 [5] D.V. Lind, et al., Prenatal exposure to perfluoroalkyl substances and anogenital  
29 distance at 3 months of age in a Danish mother-child cohort, *Reprod. Toxicol.* 68  
30 (2017) 200-206. <https://doi.org/10.1016/j.reprotox.2016.08.019>.

- 1 [6] L.L. Needham, et al., Partition of environmental chemicals between maternal and  
2 fetal blood and tissues, *Environ. Sci. Technol.* 45 (2011) 1121–1126.  
3 <https://doi.org/10.1021/es1019614>.
- 4 [7] S. Itoh, et al., Association of perfluoroalkyl substances exposure *in utero* with  
5 reproductive hormone levels in cord blood in the Hokkaido Study on  
6 Environment and Children’s Health, *Environ. Int.* 94 (2016) 51–59.  
7 <https://doi.org/10.1016/j.envint.2016.05.011>.
- 8 [8] A. Vested, et al., Associations of *in utero* exposure to perfluorinated alkyl acids with  
9 human semen quality and reproductive hormones in adult men, *Environ. Health*  
10 *Perspect.* 121 (2013) 453–458. <https://doi.org/10.1289/ehp.1205118>.
- 11 [9] F. Galis, et al., Sexual dimorphism in the prenatal digit ratio (2D:4D), *Arch. Sex.*  
12 *Behav.* 39 (2010) 57–62. <https://doi.org/10.1007/s10508-009-9485-7>.
- 13 [10] M.A. Malas, et al., Fetal development of the hand, digits and digit ratio (2D:4D),  
14 *Early Hum. Dev.* 82 (2006) 469–475.  
15 <https://doi.org/10.1016/j.earlhumdev.2005.12.002>.
- 16 [11] J.T. Manning, et al., The ratio of 2nd to 4th digit length: a predictor of sperm  
17 numbers and concentrations of testosterone, luteinizing hormone and oestrogen,  
18 *Hum. Reprod.* 13 (1998) 3000–3004. <https://doi.org/10.1093/humrep/13.11.3000>.
- 19 [12] Z. Zheng, M.J. Cohn, Developmental basis of sexually dimorphic digit ratios, *Proc.*  
20 *Natl Acad. Sci. U. S. A.* 108 (2011) 16289–16294.  
21 <https://doi.org/10.1073/pnas.1108312108>.
- 22 [13] S.H. Swan, et al., First trimester phthalate exposure and anogenital distance in  
23 newborns, *Hum. Reprod.* 30 (2015) 963–972.  
24 <https://doi.org/10.1093/humrep/deu363>.
- 25 [14] E.S. Barrett, et al., First-trimester urinary bisphenol A concentration in relation to  
26 anogenital distance, an androgen-sensitive measure of reproductive  
27 development, in infant girls, *Environ. Health Perspect.* 125 (2017) 077008.  
28 <https://doi.org/10.1289/EHP875>.
- 29 [15] J. Auger, et al., Environmental levels of oestrogenic and antiandrogenic compounds  
30 feminize digit ratios in male rats and their unexposed male progeny, *Proc. Biol.*  
31 *Sci.* 280(1768) (2013) 20131532. <https://doi.org/10.1098/rspb.2013.1532>.
- 32 [16] D.J. Macleod, et al., Androgen action in the masculinization programming window  
33 and development of male reproductive organs, *Int. J. Androl.* 33 (2010) 279–287.  
34 <https://doi.org/10.1111/j.1365-2605.2009.01005.x>.
- 35 [17] Y. Tian, et al., Maternal plasma concentrations of perfluoroalkyl and polyfluoroalkyl  
36 substances during pregnancy and anogenital distance in male infants, *Hum.*  
37 *Reprod.* 34 (2019) 1356–1368. <https://doi.org/10.1093/humrep/dez058>.

- 1 [18] T.E. Arbuckle, et al., Prenatal perfluoroalkyl substances and newborn anogenital  
2 distance in a Canadian cohort, *Reprod. Toxicol.* 94 (2020) 31-39.  
3 <https://doi.org/10.1016/j.reprotox.2020.03.011>.
- 4 [19] A.D. Benninghoff, et al., Estrogen-like activity of perfluoroalkyl acids *in vivo* and  
5 interaction with human and rainbow trout estrogen receptors *in vitro*, *Toxicol.*  
6 *Sci.* 120 (2011) 42-58. <https://doi.org/10.1093/toxsci/kfq379>.
- 7 [20] G. Du, et al., Endocrine-related effects of perfluorooctanoic acid (PFOA) in  
8 zebrafish, H295R steroidogenesis and receptor reporter gene assays,  
9 *Chemosphere.* 91 (2013) 1099-1106.  
10 <https://doi.org/10.1016/j.chemosphere.2013.01.012>, *H295R*.
- 11 [21] G. Du, et al., Perfluorooctane sulfonate (PFOS) affects hormone receptor activity,  
12 steroidogenesis, and expression of endocrine-related genes *in vitro* and *in vivo*,  
13 *Environ. Toxicol. Chem.* 32 (2013) 353-360. <https://doi.org/10.1002/etc.2034>.
- 14 [22] J. Chen, et al., Chronic perfluorooctanesulphonic acid (PFOS) exposure produces  
15 estrogenic effects in zebrafish, *Environ. Pollut.* 218 (2016) 702-708.  
16 <https://doi.org/10.1016/j.envpol.2016.07.064>.
- 17 [23] J.F. Arnal, et al., Membrane and nuclear estrogen receptor alpha actions: from  
18 tissue specificity to medical implications, *Physiol. Rev.* 97 (2017) 1045-1087.  
19 <https://doi.org/10.1152/physrev.00024.2016>.
- 20 [24] M. Ponglikitmongkol, S. Green, P. Chambon, Genomic organization of the human  
21 oestrogen receptor gene, *EMBO J.* 7 (1988) 3385-3388.  
22 <https://doi.org/10.1002/j.1460-2075.1988.tb03211.x>.
- 23 [25] I. Stavrou, et al., Association of polymorphisms of the oestrogen receptor alpha  
24 gene with the age of menarche, *Hum. Reprod.* 17 (2002) 1101-1105.  
25 <https://doi.org/10.1093/humrep/17.4.1101>.
- 26 [26] S. Ban, et al., Genetic polymorphisms of ESR1 and ESR2 that may influence  
27 estrogen activity and the risk of hypospadias, *Hum. Reprod.* 23 (2008) 1466-  
28 1471. <https://doi.org/10.1093/humrep/den098>.
- 29 [27] M.R. Safarinejad et al., Estrogen receptors alpha (rs2234693 and rs9340799), and  
30 beta (rs4986938 and rs1256049) genes polymorphism in prostate cancer:  
31 evidence for association with risk and histopathological tumor characteristics in  
32 Iranian men, *Mol. Carcinog.* 51 Suppl 1 (2012) E104-E117.  
33 <https://doi.org/10.1002/mc.21870>.
- 34 [28] H. Johansson, et al., Impact of CYP19A1 and ESR1 variants on early-onset side  
35 effects during combined endocrine therapy in the TEXT trial, *Breast Cancer Res.*  
36 18 (2016) 110. <https://doi.org/10.1186/s13058-016-0771-8>.

- 1 [29] Y. Nishimura, et al., Association between ESR1 polymorphisms and second to  
2 fourth digit ratio in school-aged children in the Hokkaido Study, *Steroids*. 141  
3 (2019) 55–62. <https://doi.org/10.1016/j.steroids.2018.11.011>.
- 4 [30] Y. Nishimura, et al., Association of exposure to prenatal phthalate esters and  
5 bisphenol A and polymorphisms in the ESR1 gene with the second to fourth digit  
6 ratio in school-aged children: data from the Hokkaido study, *Steroids*. 159 (2020)  
7 108637. <https://doi.org/10.1016/j.steroids.2020.108637>.
- 8 [31] R. Kishi, et al., Cohort profile: the Hokkaido study on environment and children's  
9 health in Japan, *Int. J. Epidemiol.* 40 (2011) 611–618.  
10 <https://doi.org/10.1093/ije/dyq071>.
- 11 [32] R. Kishi, et al., Ten years of progress in the Hokkaido birth cohort study on  
12 environment and children's health: cohort profile--updated 2013, *Environ. Health*  
13 *Prev. Med.* 18 (2013) 429–450. <https://doi.org/10.1007/s12199-013-0357-3>.
- 14 [33] R. Kishi, et al., The Hokkaido Birth Cohort Study on Environment and Children's  
15 Health: cohort profile--updated 2017, *Environ. Health Prev. Med.* 22 (2017) 46.  
16 <https://doi.org/10.1186/s12199-017-0654-3>.
- 17 [34] E. Okada, et al., Temporal trends of perfluoroalkyl acids in plasma samples of  
18 pregnant women in Hokkaido, Japan, 2003–2011, *Environ. Int.* 60 (2013) 89–96.  
19 <https://doi.org/10.1016/j.envint.2013.07.013>.
- 20 [35] H. Goudarzi, et al., Prenatal exposure to perfluorinated chemicals and  
21 neurodevelopment in early infancy: the Hokkaido Study, *Sci. Total Environ.* 541  
22 (2016) 1002–1010. <https://doi.org/10.1016/j.scitotenv.2015.10.017>.
- 23 [36] J. Jurečková, et al., Estrogen receptor alpha polymorphisms and the risk of  
24 prostate cancer development, *J. Cancer Res. Clin. Oncol.* 141 (2015) 1963–1971.  
25 <https://doi.org/10.1007/s00432-015-1966-6>.
- 26 [37] L.S. Kjeldsen, E.C. Bonefeld-Jørgensen, Perfluorinated compounds affect the  
27 function of sex hormone receptors, *Environ. Sci. Pollut. Res. Int.* 20 (2013) 8031–  
28 8044. <https://doi.org/10.1007/s11356-013-1753-3>.
- 29 [38] Z. Qiu, et al., Binding specificities of estrogen receptor with perfluorinated  
30 compounds: A cross species comparison, *Environ. Int.* 134 (2020) 105284.  
31 <https://doi.org/10.1016/j.envint.2019.105284>.
- 32 [39] Q. Yao, et al., Cord blood Per- and polyfluoroalkyl substances, placental  
33 steroidogenic enzyme, and cord blood reproductive hormone, *Environ. Int.* 129  
34 (2019) 573–582. <https://doi.org/10.1016/j.envint.2019.03.047>.
- 35 [40] A.J. Andrade, et al., A dose–response study following *in utero* and lactational  
36 exposure to *di*-(2-ethylhexyl)-phthalate (DEHP): non-monotonic dose–response

- 1 and low dose effects on rat brain aromatase activity, *Toxicology*. 227 (2006) 185–  
2 192. <https://doi.org/10.1016/j.tox.2006.07.022>.
- 3 [41] C.E. Roselli, et al., Sex differences in androgen-regulated cytochrome P450  
4 aromatase mRNA in the rat brain, *Endocrine*. 5 (1996) 59–65.  
5 <https://doi.org/10.1007/BF02738657>.
- 6 [42] Y. Li, et al., Expression of 3beta-hydroxysteroid dehydrogenase type 1, P450  
7 aromatase, and 17beta-hydroxysteroid dehydrogenase types 1, 2, 5 and 7 mRNAs  
8 in human early and mid-gestation placentas, *Placenta*. 26 (2005) 387–392.  
9 <https://doi.org/10.1016/j.placenta.2004.07.008>.
- 10 [43] J.S. Kang, J.S. Choi, J.W. Park, Transcriptional changes in steroidogenesis by  
11 perfluoroalkyl acids (PFOA and PFOS) regulate the synthesis of sex hormones in  
12 H295R cells, *Chemosphere*. 155 (2016) 436–443.  
13 <https://doi.org/10.1016/j.chemosphere.2016.04.070>.
- 14 [44] M. Welsh, et al., Identification in rats of a programming window for reproductive  
15 tract masculinization, disruption of which leads to hypospadias and  
16 cryptorchidism, *J. Clin. Invest.* 118 (2008) 1479–1490.  
17 <https://doi.org/10.1172/JCI34241>.
- 18 [45] Z. Shi, et al., Chronic exposure to perfluorododecanoic acid disrupts testicular  
19 steroidogenesis and the expression of related genes in male rats, *Toxicol. Lett.*  
20 188 (2009) 192–200. <https://doi.org/10.1016/j.toxlet.2009.04.014>.
- 21 [46] Z. Shi, et al., The effect of perfluorododecanoic acid on endocrine status, sex  
22 hormones and expression of steroidogenic genes in pubertal female rats, *Reprod.*  
23 *Toxicol.* 27 (2009) 352–359. <https://doi.org/10.1016/j.reprotox.2009.02.008>.
- 24 [47] O.R. Ibor, et al., Contaminant levels and endocrine disruptive effects in *Clarias*  
25 *gariepinus* exposed to simulated leachate from a solid waste dumpsite in  
26 Calabar, Nigeria, *Aquat. Toxicol.* 219 (2020) 105375.  
27 <https://doi.org/10.1016/j.aquatox.2019.105375>.
- 28 [48] S. Sathyanarayana, et al., A pilot study of the association between genetic  
29 polymorphisms involved in estrogen signaling and infant male genital  
30 phenotypes, *Asian J. Androl.* 14 (2012) 766–772.  
31 <https://doi.org/10.1038/aja.2012.27>.
- 32 [49] S. Rehman, et al., Endocrine disrupting chemicals and impact on male reproductive  
33 health, *Transl. Androl. Urol.* 7 (2018) 490–503.  
34 <https://doi.org/10.21037/tau.2018.05.17>.
- 35 [50] C.A. Harris, et al., The estrogenic activity of phthalate esters *in vitro*, *Environ.*  
36 *Health Perspect.* 105 (1997) 802–811. <https://doi.org/10.1289/ehp.97105802>.

- 1 [51] S. Takeuchi, et al., Differential effects of phthalate esters on transcriptional  
2 activities via human estrogen receptors alpha and beta, and androgen receptor,  
3 Toxicology. 210 (2005) 223–233. <https://doi.org/10.1016/j.tox.2005.02.002>.
- 4 [52] A.C. Behr, et al., Perfluoroalkylated substances (PFAS) affect neither estrogen and  
5 androgen receptor activity nor steroidogenesis in human cells *in vitro*, Toxicol.  
6 Lett. 291 (2018) 51–60. <https://doi.org/10.1016/j.toxlet.2018.03.029>.
- 7 [53] J. Li, et al., Evaluation of the estrogenic/antiestrogenic activities of perfluoroalkyl  
8 substances and their interactions with the human estrogen receptor by  
9 combining In Vitro Assays and In Silico Modeling, Environ. Sci. Technol. 54  
10 (2020) 14514–14524. <https://doi.org/10.1021/acs.est.0c03468>.
- 11 [54] U.N. Joensen, et al., PFOS (perfluorooctanesulfonate) in serum is negatively  
12 associated with testosterone levels, but not with semen quality, in healthy men,  
13 Hum. Reprod. 28 (2013) 599–608. <https://doi.org/10.1093/humrep/des425>.
- 14 [55] G. Toft, et al., Exposure to perfluorinated compounds and human semen quality in  
15 Arctic and European populations, Hum. Reprod. 27 (2012) 2532–2540.  
16 <https://doi.org/10.1093/humrep/des185>.
- 17 [56] A.L. Brantsæter, et al., Determinants of plasma concentrations of perfluoroalkyl  
18 substances in pregnant Norwegian women, Environ. Int. 54 (2013) 74–84.  
19 <https://doi.org/10.1016/j.envint.2012.12.014>.
- 20 [57] M.S. Tsai, et al., Determinants and temporal trends of perfluoroalkyl substances in  
21 pregnant women: the Hokkaido study on environment and children’s health, Int.  
22 J. Environ. Res. Public Health. 15 (2018).  
23 <https://doi.org/10.3390/ijerph15050989>.

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