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

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2D:4D, ratio of the lengths of the second and fourth digits; AGD, anogenital distance; CI, confidence interval; CYP19, aromatase cytochrome P450; E_2, estradiol; EDC, endocrine-disrupting chemical; ER, estrogen receptor; ESR1, estrogen receptor 1; MDL, method detection limit; OCC, Odense Child Cohort; PFAS, perfluoroalkyl substances; PFHpA, perfluoroheptanoic acid; PFHxA, perfluorohexanoic acid; PFHxS, perfluorohexane sulfonate; PFDA, perfluorodecanoic acid; PFDoDA, perfluorododecanoic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PFTeDA, perfluorotetradecanoic acid; PFTrDA, perfluorotridecanoic acid; PFUnDA, perfluoroundecanoic acid; SNP,

single-nucleotide polymorphism; T_2, testosterone; MEHP, mono(2-ethylhexyl) phthalate; DEHP, di(2-ethylhexyl) phthalate.

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ABSTRACT

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

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

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

1. Introduction

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

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

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

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

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

In vivo and *in vitro* experiments have demonstrated that PFAS can bind to ERs and exhibit environmental xenoestrogenic activity [19]. Moreover, animal studies have shown that PFAS exposure increases estradiol (E_2) production and decreases testosterone (T_2) production [20-22]. Furthermore, exposure to PFAS results in higher expression of the estrogen receptor 1 gene (*ESR1*) [20-22], encoding ERα, which plays a key role in both reproductive and nonreproductive tissues in the human body [23,24]. *ESR1* harbors two well-known, functional, single-nucleotide polymorphisms (SNPs) [*PvuII* (T>C, dbSNP: rs2234693) and *XbaI* (A>G, dbSNP: rs9340799)], along with the silent polymorphism rs2077647 (A>G). These polymorphisms are associated with menarche, breast cancer, prostate cancer, and hypospadias [25-28]. We previously reported that *ESR1* polymorphisms are related to 2D:4D [29] and alter the association between prenatal exposure to di(2-ethylhexyl) phthalate (DEHP) and 2D:4D in 7-year-old children [30]. It is possible that *ESR1*

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

2. Material and methods

2.1. Participants and data collection

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

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

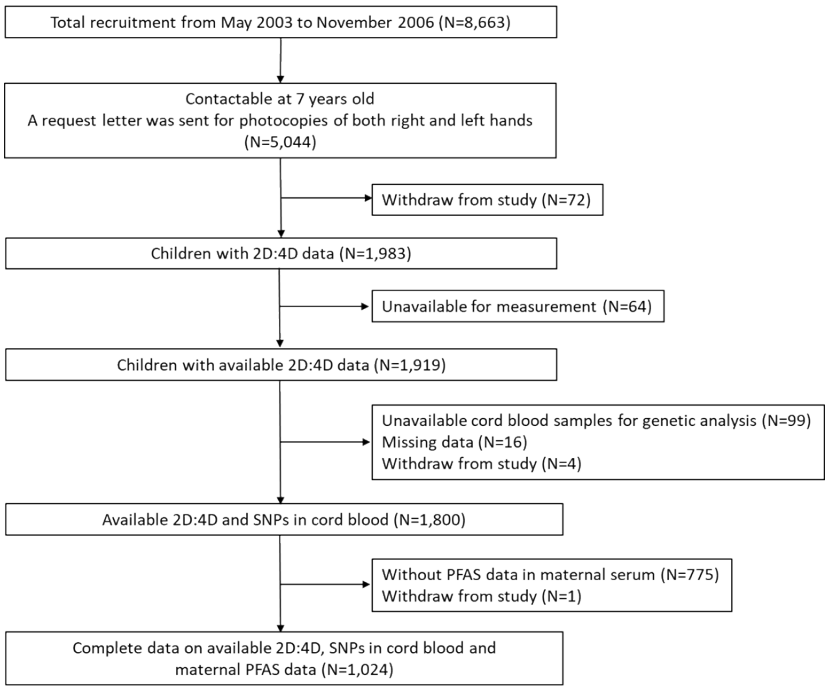

Fig. 1. Flow chart of participant selection from the Hokkaido Study. 2D:4D, ratio of the lengths of the second and fourth digits in human hands; SNP, single-nucleotide polymorphism; PFAS, perfluoroalkyl substances.

2.2. PFAS concentration measurement

Blood samples had been collected at 25 to 41 weeks of gestation (median: 29 weeks of pregnancy) and stored at −80 °C until analysis. We analyzed the concentration of 11 PFAS in maternal plasma samples, namely perfluorohexane sulfonate (PFHxS), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOS, PFOA,

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

2.3. ESR1 genotyping in children

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

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

2.4. 2D:4D measurement

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

2.5. Statistical analysis

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

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

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

2.6. Ethical considerations

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

3. Results

3.1. Cohort characteristics

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

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

Table 1. Participant characteristics (n = 1,024)

	n (%) or mean ± SD
Maternal characteristics	
Age at delivery (year)	31.1 ± 4.2
Pre-pregnancy body mass index (kg/m^2)	20.9 ± 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)
≥ 5	353 (34.5)
Missing data	117 (11.4)
Education level (year)	
< 12	397 (38.8)
≥ 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)
Children characteristics	
Sex	
Male	513 (50.1)
Female	511 (49.9)
Birth weight (g)	3,059 ± 365
Gestational age (weeks)	38.8 ± 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)

3.2. PFAS concentration in maternal serum after stratification by ESR1 polymorphisms in children

Supplemental Table S1 shows the distribution of the levels of 11 PFAS. PFOS had the highest median concentration (6.06 ng/mL), followed by PFOA (1.98 ng/mL), PFUnDA (1.36 ng/mL), PFNA (1.06 ng/mL), PFDA (0.50 ng/mL), PFHxS (0.31 ng/mL), PFTrDA (0.33 ng/mL), and PFDoDA (0.17 ng/mL). There were no significant differences in exposure levels based on sex (data not shown). Supplemental Table S3 shows the median (interquartile range) concentration of each PFAS stratified by *ESR1* polymorphisms. There were no significant differences in the median concentration of PFAS with respect to the *ESR1* genotype.

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

Supplemental Table S4 shows the associations between *ESR1* polymorphisms and 2D:4D. In dominant models, children with an *ESR1* polymorphism did not show a significant difference in the percentage change in 2D:4D compared with children in the reference group. Furthermore, the results of the analysis indicated no association between *ESR1* polymorphisms and digit ratio.

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

Table 2 shows the association between the ($\log_{10}$-transformed) PFAS concentration and 2D:4D. A 10-fold increase in the maternal concentration of PFAS was associated with a non-significant but increasing trend in 2D:4D in children, except for PFDA, which was associated with a non-significant but decreasing trend in 2D:4D. Particularly, a 10-fold increase in the maternal PFOA concentration was associated with a 0.88% increase (95% CI: −0.02, 1.78) in 2D:4D in all participants; however, only males showed a statistically significant

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

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

	All [a]	Male [b]	Female [b]
	β (95% CI)	β (95% CI)	β (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)
PFTrDA	0.44 (−0.26, 1.34)	0.66 (−0.59, 1.90)	0.21 (−1.12, 1.54)

[a] Multiple linear regression adjusted for sex, birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.
[b] Multiple linear regression adjusted for birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.
Because the PFAS levels were $\log_{10}$-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; PFHxS, perfluorohexane sulfonate; PFDA, perfluorodecanoic acid; PFDoDA, perfluorododecanoic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; PFTrDA, perfluorotridecanoic acid; PFUnDA, perfluoroundecanoic acid.

3.5. Effect of gene-environment interactions between ESR1 polymorphisms and PFAS on 2D:4D

Gene-environment interactions between ($\log_{10}$-transformed) PFAS and *ESR1* polymorphisms are shown in Table 3. With respect to the effect of

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

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

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

	All [a]	Male [b]	Female [b]
	β (95% CI)	β (95% CI)	β (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 × rs2234693-TT	0.66 (−0.25, 1.56)	0.85 (−0.41, 2.12)	0.63 (−0.71, 1.97)
	P_{int} = 0.157	P_{int} = 0.186	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 × rs9340799-AA	1.03 (0.15, 1.90)*	1.08 (−0.10, 2.25)	0.93 (−0.39, 2.26)
	P_{int} = 0.022	P_{int} = 0.073	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 × rs2077647-AA	1.06 (0.17, 1.95)*	1.35 (0.12, 2.58)*	1.01 (−0.30, 2.33)
	P_{int} = 0.020	P_{int} = 0.032	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 × rs2234693-TT	0.27 (−0.70, 1.24)	1.22 (−0.20, 2.64)	−0.55 (−1.92, 0.81)
	P_{int} = 0.588	P_{int} = 0.093	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)
PFDoA × 9340799-AA	0.87 (−0.09, 1.83)	1.31 (−0.03, 2.65)	0.46 (−0.95, 1.86)
	P_{int} = 0.077	P_{int} = 0.056	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

[a] Multiple linear regression adjusted for sex, birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.
[b] Multiple linear regression adjusted for birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.
Because the PFAS levels were $\log_{10}$-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.

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

Table 4 shows the association between the ($\log_{10}$-transformed) PFAS concentrations and 2D:4D stratified by *ESR1* polymorphisms. A 10-fold increase in the maternal PFOA concentration was associated with a 1.54% increase (95% CI: 0.40, 2.68) in 2D:4D in children with the AA genotype of rs9340799, whereas children with the AG/GG genotype showed no significant change in 2D:4D. A 10-fold increase in PFOA concentration was associated with a 2.24% increase (95% CI: 0.57, 3.92) in 2D:4D in children with the AA genotype at rs2077647, whereas children with the AG/GG genotype showed no significant change in 2D:4D. These associations were only apparent among males when the data were stratified by sex [rs9340799, 2.17% (95% CI: 0.66, 3.68);

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

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

Exposure		Genotype model	All[a] β (95% CI)	Male[b] β (95% CI)	Female[b] β (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)

[a] Multiple linear regression adjusted for sex, birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.
[b] Multiple linear regression adjusted for birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.
Because the PFAS levels were $\log_{10}$-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.

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

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

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

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

Exposure		Genotype model	All[a] β (95% CI)	Male[b] β (95% CI)	Female[b] β (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)

[a] Multiple linear regression adjusted for sex, birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.
[b] Multiple linear regression adjusted for birth weight, maternal age, parity, alcohol consumption, and smoking in the first trimester.
β (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.

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

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

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

4. Discussion

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

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

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

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

Previous *in vitro* studies have shown that PFOA exerts agonistic activity via ERα, through which it exerts its estrogenic activity [19], significantly induces ER transactivity [37], and promotes *ESR1* expression [20]. A study on rats demonstrated that PFOA exposure increased ERα expression [38], and cord blood PFOA levels were shown to be positively associated with E_2 levels in human fetuses [39]. These findings suggest that PFOA exposure might activate ERα and inhibit fourth digit development in children, eventually causing feminized 2D:4D.

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

aromatase cytochrome P450 gene (*CYP19*), which encodes aromatase, can increase in response to PFOA exposure. An *in vitro* study showed that PFOA exposure significantly induced CYP19 expression [20], increased E_2 production, and decreased T_2 production [20,43]. Aromatase activity was reportedly enhanced with PFOS exposure in human male infants [7]. Therefore, PFOA might enhance aromatase activity, and males with a higher PFOA exposure would have a higher estrogen supply than males with a lower exposure. Additionally, the evaluation of feminized effects in females is challenging, since a human female fetus develops under androgen deficiency [44]. In females, 2D:4D showed an increasing trend with respect to the association or interaction terms between PFOA and *ESR1* polymorphism. However, the effect sizes were smaller and non-significant in females, in contrast to the larger and statistically significant effect sizes in males. Therefore, the feminized effects on 2D:4D in females may be negligible.

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

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

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

activity [26]; therefore, children with the AA genotype in rs9340799 exposed to PFAS might show greater ERα activity. However, children with the AA genotype at rs2077647, which has been hypothesized to inhibit ERα activity [48], showed feminized 2D:4D with exposure to PFAS. This result was inconsistent with our expectation that children with the G allele would show feminized 2D:4D. Although the specific reasons underlying this inconsistency remain unclear, possibly, rs2077647 polymorphisms are in linkage disequilibrium with other polymorphisms that affect *ESR1* function or PFAS metabolism. Additionally, there could be an affinity between PFAS and the A allele of rs2077647; however, additional studies are needed to confirm this, as there are no reports on the affinity between PFAS and specific *ESR1* polymorphisms. Although we observed a significant P_{int} between PFNA and rs9340799, we believe that this result was coincidental, because analysis between 2D:4D and PFNA exposure stratified by the rs9340799 polymorphism did not reveal any significant differences.

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

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

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

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

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

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

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

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

5. Conclusion

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

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

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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