# Association of perfluoroalkyl substances exposure in utero with reproductive hormone levels in cord blood in the Hokkaido Study on Environment and Children's Health

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**Abbreviations**

PFASs; perfluoroalkyl substances

PFOS; perfluorooctane sulfonate

PFOA; perfluorooctanoate

E2; estradiol

T; testosterone

P4; progesterone

LH; luteinizing hormone

FSH; follicle-stimulating hormone

SHBG; steroid hormone binding globulin

PRL; prolactin

INSL3; insulin-like factor 3

**Abstract**

*BACKGROUND*: Exposure to perfluoroalkyl substances (PFASs) may disrupt reproductive function in animals and humans. Although PFASs can cross the human placental barrier, few studies evaluated the effects of prenatal PFAS exposure on the fetus' reproductive hormones.

*OBJECTIVE*: To explore the associations of prenatal exposure to perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) with cord blood reproductive hormones.

*METHODS*: In the prospective birth cohort (Sapporo cohort of the Hokkaido study), we included 189 mother-infant pairs recruited in 2002-2005 with both prenatal maternal and cord blood samples. PFOS and PFOA levels in maternal blood after the second trimester were measured via liquid chromatography-tandem mass spectrometry. We also measured cord blood levels of the fetuses' reproductive hormones, including estradiol (E2), total testosterone (T), progesterone (P4), inhibin B, insulin-like factor 3 (INSL3), steroid hormone binding globulin (SHBG), follicle-stimulating hormone, and luteinizing hormone, and prolactin (PRL).

*RESULTS*: The median PFOS and PFOA levels in maternal serum were 5.2 ng/mL and 1.4 ng/mL, respectively. In the fully adjusted linear regression analyses of the male

infants, maternal PFOS levels were significantly associated with E2 and positively, and T/E2, P4, and inhibin B inversely; PFOA levels were positively associated with inhibin B levels. Among the female infants, there were significant inverse associations between PFOS levels and P4 and PRL levels, although there were no significant associations between PFOA levels and the female infants' reproductive hormone levels.

*CONCLUSIONS*: These results suggest that the fetal synthesis and secretion of reproductive hormones may be affected by in utero exposure to measurable levels of PFOS and PFOA.

**Keywords**

Perfluoroalkyl substances, reproductive hormones, cord blood, prenatal exposure, birth cohort

## 1. Introduction

Perfluoroalkyl substances (PFASs) are widely-used in industrial products and are commonly detected in the environment. Human exposure to PFASs mainly occurs orally, via the intake of contaminated food, water, and dust (Fromme et al., 2009). As perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) are the most commonly detected PFASs in the environment and in humans, their presence in human blood has been reported in several countries (Butenhoff et al., 2006; Calafat et al., 2007; Harada et al., 2007; Midasch et al., 2007). Furthermore, in 2009, PFOS was added to Annex B of the Stockholm Convention on Persistent Organic Pollutants. PFOA is now proposed to be listed on Stockholm Convention by EU. Although PFOS and PFOA are being voluntarily phased out by several industries, they are still present in older products and have long elimination half-lives in human serum (PFOS: 5.3 years, PFOA: 3.8 years) (Olsen et al., 2007). PFOS and PFOA can cross the placental barrier, and be transferred from mother to fetus in humans (Inoue et al., 2004; Midasch et al., 2007). Therefore, significant concern has been raised regarding the adverse effects of in utero exposure to PFOS and PFOA on the fetus.

Epidemiological studies have reported that prenatal exposures to PFOS and PFOA were inversely associated with birth size and neurodevelopment during

childhood (Apelberg et al., 2007; Chen et al., 2013). Our group, the Hokkaido Study on Environment and Children's Health, has also reported an inverse association between maternal PFOS serum levels and birth weight among female infants (Washino et al., 2009). Moreover, we found inverse associations of PFOS with essential fatty acid and triglyceride serum levels in pregnant mothers (Kishi et al., 2015), at levels of PFOS and PFOA that were lower than those found in other countries and areas in Japan (Okada et al., 2013).

Recent studies have suggested that PFASs can disrupt the endocrine system, such as levels of reproductive hormones, toxicity on reproductive cells and gene expression. For example, PFOS and PFOA treatment in adult mice caused decreases in serum testosterone (T) and epididymal sperm counts (Wan et al., 2011), and an increase in progesterone (P4) levels (Zhao Y et al., 2010). In addition, Zhao et al. (2014) have reported that PFOS exposure was associated with a reduced number of fetal Leydig cells, reduced steroidogenic enzyme gene expression, and lower T levels in pregnant rats. As for human populations, a cross-sectional study in children at 6-9 years of age in Mid-Ohio Valley reported that PFAS concentrations inversely associated with serum levels of T, estradiol (E2) and insulin-like growth factor-1 (IGF-1) (Lopez-Espinosa et al., 2016). A study among Taiwanese at 12-17 years of age also reported the

associations of PFAS with lowered T, follicle stimulating hormone (FSH) and sex-hormone binding globulin (SHBG) levels (Tsai et al., 2015). In adult populations, some cross-sectional reports have also revealed associations between serum PFAS concentrations and altered serum levels of E2, T, and luteinizing hormone (LH) (Joensen et al., 2009; Joensen et al., 2013; Knox et al., 2011; Raymer et al., 2012). Furthermore, two prospective studies have reported that PFOS and PFOA exposures in utero were positively associated with serum T levels among young girls (Maisonet et al., 2015), and that PFOA exposure in utero was positively associated with LH and FSH levels, and inversely associated with sperm concentrations and total sperm counts among adult men (Vested et al., 2013). Those studies appear to indicate that prenatal exposure to PFOS and PFOA disrupts reproductive functions by altering reproductive hormone secretions at a later age.

Prenatal period is very important for the growth of fetuses. Disrupted endocrine environment during the perinatal period is considered to influence on the reproductive health in adulthood (Lagiou et al., 2011). Although reproductive issues among adults may have fetal origins (Crain et al., 2008; Juul et al., 2014), there is limited numbers of studies investigating the PFAS impact on fetal reproductive health. It is necessary to clarify the effects of PFAS exposure during the fetal period. Therefore, the present study

aimed to investigate the effects of prenatal exposure to PFOS and PFOA on fetal reproductive hormones.

## 2. Materials and Methods

### 2.1 Participants

This prospective birth cohort study was based on the Hokkaido Study on Environment and Children's Health. Details regarding the study population, data collection, biological specimen sampling, and questionnaire's contents have been described elsewhere (Kishi et al., 2013; Kishi et al., 2011). In brief, native Japanese citizens who resided in Sapporo or its suburbs were recruited at 23–35 weeks of gestation between July 2002 and October 2005 at Sapporo Toho Hospital (Sapporo, Hokkaido, Japan). Of 1796 potentially eligible pregnant women, the following women were excluded: decided to participate in the Japanese cord blood bank (22% of those approached), delivered at another hospital (3% of those approached). Of the remaining eligible subjects, 514 (28.6% of those approached) pregnant women agreed to participate in this study. All participants provided written informed consent before the participation in the Hokkaido Study and the study protocol was approved by the ethics review board for epidemiological studies at Hokkaido University Graduate School of

159 Medicine and the Hokkaido University Center for Environmental and Health Sciences.
160 This study was performed in accordance with principles of the Declaration of Helsinki.
161 Among the 514 participants, we excluded women who experienced miscarriage,
162 stillbirth, relocation, or voluntary withdrawal (n = 10), and women who delivered twins
163 (n = 7). 447 maternal blood samples were available for the PFOS and PFOA
164 measurements, and 295 cord blood samples were available for the reproductive
165 hormone measurements. In addition, among 257 participants with paired maternal and
166 cord blood samples, maternal blood samples form 68 women were obtained after their
167 delivery due to anemia. Since these post-delivery blood samples exhibited significantly
168 lower PFOS and PFOA (before delivery: PFOS = 5.2 and PFOA = 1.4 ng/mL;
169 post-delivery: PFOS = 3.5 ng/mL and PFOA = 1.3 ng/mL), those 68 women were
170 excluded. Finally, 189 mother-infant pairs were included in the statistical analysis.
171 **2.2 Exposure assessment**
172 We used the data of PFOS and PFOA concentrations in maternal serum
173 measured by liquid chromatography-tandem mass spectrometry (LC/MS/MS) in our
174 previous study. Detailed methods for the measurement of PFOS and PFOA have been
175 described in our previous reports (Kishi et al., 2015; Nakata A, 2009). 6.9% of the
176 samples exhibited PFOA levels that were below the limit of detection (LOD; 0.50

177 ng/mL). In those cases, we assigned the sample a value of 0.25 ng/mL (50% of the
178 LOD).

179 **2.3 Outcome measures**

180 A blood sample (10–30 mL) was collected from the umbilical cord at the time
181 of delivery and was stored at –80°C until analysis. We analyzed cord blood samples for
182 their levels of E2, T, P4, LH, FSH, SHBG, prolactin (PRL), and inhibin B, as described
183 in our previous report (Araki et al., 2014). The concentration of insulin-like factor 3
184 (INSL3) was measured for all male infants using an enzyme immunoassay (INSL3 /
185 RLF [human] EIA kit; Phoenix Pharmaceuticals Inc., CA, USA). However, due to the
186 low detection rate among female infants in previous studies (Bay et al., 2007;
187 Szydlarska et al., 2012), we only tested 25 female infants for INSL3. All reproductive
188 hormone measurements were performed at Asuka Pharma Medical Co. Ltd (Kanagawa,
189 Japan). Samples that exhibited values below the LOD of the test were assigned a value
190 that was 50% of that test's LOD.

191 **2.4 Questionnaire and medical records**

192 All participants completed a self-administered questionnaire at enrollment
193 regarding their maternal age, educational level, household income, smoking status and
194 medical history. Maternal smoking status during pregnancy was classified as

non-smoker (never smoked or quit smoking during the first trimester) and smoker (had smoked after the first trimester). Medical records were obtained to collect information regarding body mass index (BMI) before pregnancy, pregnancy complications, gestational age, infant sex, parity, congenital anomalies (including hypospadias and cryptorchidism), and infant birth sizes.

**2.5 Statistical analyses**

The associations of PFOS and PFOA with the maternal and fetal characteristics were explored using Spearman's correlation test and the Mann-Whitney U test. Correlations of maternal serum PFOS and PFOA levels with cord blood reproductive hormone levels were analyzed using Spearman's correlation test. We also performed two different analyses: multiple linear regression analysis and dose-response analysis. In the linear regression analysis, levels of PFOS, PFOA, and reproductive hormones were converted using a log10 scale to account for their skewed distribution (Supplemental Figure 1, 2). In the dose-response analyses, PFOS and PFOA levels were categorized into four quartiles, and reproductive hormones levels were converted using a log10 scale. The least square means of each reproductive hormone were calculated and back transformed. The p-value for trend was calculated using linear contrast coefficient –3, –1, +1, +3 assigned to quartile first, second, third and fourth, respective

213 (Goudarzi et al., 2016; Kishi et al., 2015). The first quartile was also compared to the
214 second, third, and fourth quartiles of PFOS and PFOA, and the p-values were calculated
215 using Hsu-Dunnett's test. In the fully adjusted model, potential confounders were
216 selected based on the existing literature and the results of the present study ($p < 0.05$ in
217 Table 1; infant factor: gestational age at birth [weeks] [continuous variable], maternal
218 factors: age at delivery [years], parity [0/≥1], BMI before pregnancy [kg/m$^2$], annual
219 income (<5/≥5 million yen], smoking during pregnancy [yes/no], caffeine consumption
220 during pregnancy (mg/day), and gestational weeks of blood sampling for the PFOS and
221 PFOA measurements [continuous variable]). We also performed sensitivity analyses
222 and conducted the backward procedure to exclude some confounders, and compared to
223 the final models. As for BMI, no correlation was found between maternal BMI and
224 hormone levels in cord blood. However, we selected BMI as one of confounders
225 according to the associations between obesity and hormone levels during pregnancy
226 (Lassance et al., 2015). Because of association of steroid hormone levels in fetal serum
227 to gestational age (Procianoy and de Oliveira-Filho, 1996), we included it as a
228 confounder. We also included annual income as an indicator of socioeconomic status
229 into the final models. All statistical analyses were performed using JMP Pro software
230 (version 12; SAS Institute Inc., NC, USA).

## 3. Results

Among the 189 mother-infant pairs, the median maternal levels of PFOS and PFOA were 5.2 ng/mL (interquartile range [IQR]: 3.85–7.15 ng/mL) and 1.4 ng/mL (IQR: 0.90–2.20 ng/mL), respectively. Table 1 shows the maternal serum PFOS and PFOA levels in relation to the maternal and infants' characteristics; PFOS and PFOA levels were significantly higher among primiparous women. More than 20% of women had smoked during pregnancy, and PFOS level was significantly lower among smokers. The amount of caffeine intake was negatively associated with PFOA levels. The mean of the gestational weeks of blood sampling for the PFOS and PFOA measurement was 33.3 weeks. PFOS and PFOA levels were negatively correlated with increasing gestational weeks of pregnancy. Maternal PFOS was significantly correlated with the birth weight of female infants.

The cord blood reproductive hormone levels are shown in Table 2. Less than 30% of female infants exhibited detectable levels of LH, FSH, and inhibin B. Therefore, we omitted those hormones from further analyses in the female infants. Furthermore, because male infants exhibited significantly higher median levels of T, LH, FSH, inhibin B, and INSL3 (vs. female infants), we performed all further analyses after

249 stratifying the data according to infant sex.

250 Table 3 shows the correlations of maternal PFOS and PFOA levels with cord
251 blood reproductive hormone levels. In the male infants, PFOS levels were positively
252 correlated with E2, and were inversely correlated with the T/E2 ratio and inhibin B
253 levels. Maternal PFOA levels were positively correlated with male P4 and inhibin B
254 levels. In the female infants, maternal PFOS was inversely correlated with PRL levels.

255 The relationships between maternal PFOS and PFOA levels and reproductive
256 hormone concentrations, according to our linear regression analyses for male and
257 female infants are shown in Table 4 and Table 5, respectively. After fully adjusting for
258 the potential confounders, maternal PFOS exhibited significant positive association with
259 E2 in male infants (β = 0.372; 95% confidence interval [CI]: 0.057, 0.687; p = 0.021),
260 and negative associations with the male infants' values for the T/E2 ratio (β = –0.399;
261 95% CI: –0.643, –0.156; p = 0.008), P4 (β = –0.344; 95% CI: –0.678, –0.010; p =
262 0.043), and inhibin B (β = –0.439; 95% CI: –0.620, –0.257; p < 0.001). Among the male
263 infants, maternal PFOA was positively associated with inhibin B (β = 0.197; 95% CI:
264 0.009, 0.384; p = 0.040). Similarly, among the female infants, we observed negative
265 associations between maternal PFOS and P4 (β = –0.552; 95% CI: –0.894, –0.210; p =
266 0.002), and PRL (β = –0.491; 95% CI: –0.764, –0.218; p = 0.001), although PFOA was

not significantly associated with any hormone levels.

We also investigated the dose-response relationships between the quartiles of maternal PFOS and PFOA levels and cord blood reproductive hormone levels (using factors in Table 4 and 5 with a p-value of <0.1). Figure 1 shows the hormones in male infants with a significant p-value for trend (<0.05). After fully adjusting for the confounders, these analyses revealed increasing trend for E2 (A), decreasing trends for the T/E2 ratio (B), and inhibin B levels (D) in relation to the PFOS quartiles. The analyses among female infants are shown in Figure 2. Significant decreasing trends were observed for P4 (A) and PRL levels (B) in relation to the PFOS quartiles.

## 4. Discussion

Reproductive hormones in prenatal period could have permanent effects on the differences in reproductive structure, behavior and cognition between males and females (Collaer and Hines, 1995). Therefore, it is important to examine the disruptive effects of PFAS exposure on reproductive hormone balances during fetal period. To our knowledge, this is the first study to report the associations between prenatal PFAS exposures and cord blood reproductive hormone levels. Our analysis of 8 hormones (including steroid, peptide, and pituitary hormones) and 1 binding protein allow us to

evaluate the PFAS effects on not only changes of each hormone level but also hormone balances. In the present study, we found that maternal PFOS levels were significantly and positively associated with E2 and inversely associated with T/E2, P4, and inhibin B in male's cord blood, and that maternal PFOA levels were positively associated with the male infants' levels of inhibin B. Among the female infants, we found that maternal PFOS levels were significantly and inversely associated with P4 and PRL levels, and that there were no significant associations between maternal PFOA levels and female infants' reproductive hormones levels.

There are some previous studies that evaluated the associations between in utero PFOS and PFOA exposure and adolescents' reproductive hormone levels. As for the exposure levels, a UK study reported median levels for PFOS and PFOA of 19.2 ng/mL and 3.6 mg/mL in the median pregnancy week 16 between 1991 and 1992, respectively (Maisonet et al., 2015), and a Danish study reported median levels of 21.2 ng/mL and 3.8 mg/mL in pregnancy week 30 in 1988-1989, respectively (Vested et al., 2013). In this study, maternal blood samples were obtained during 2002-2005 and the median gestational week of blood sampling for PFOS and PFOA was 33.3 weeks. Median levels of PFOS and PFOA are 5.2 ng/mL and 1.4 ng/mL, respectively, which were lower than in other areas of Japan (Harada et al., 2010) and in other previous

303 studies. Our results suggest that even low levels of PFOS and PFOA exposure can
304 disrupt reproductive hormone imbalance in the fetus. It is noted that the percentage of
305 smoking women in Hokkaido tend to be higher compared to other area in Japan
306 (Matsuzaki et al., 2014). Table 1 shows that 21.7 % of our participants continued
307 smoking during pregnancy, which was not significantly different from participants in
308 original cohort (n=514) (Supplemental Table 1). The level of PFOS among smoking
309 women was significantly lower than that of non-smoking women. Our result is
310 consistent with previous study.

311 There is few study that investigated the levels of reproductive hormone levels
312 in cord blood. Regarding the comparison of those levels in cord blood, our data in Table
313 2 did not differ dramatically from previous studies (Hollier et al., 2014; Kuijper et al.,
314 2013; Warembourg et al., 2016).

315 Among the male infants, we observed that maternal PFOS levels were
316 significantly and inversely associated with P4 levels and the T/E2 ratio, while
317 associated with increased E2 levels. We examined the T/E2 ratio as a marker of
318 aromatase activity, which convert T to E2 (Simpson et al., 1994). The fetal adrenal uses
319 the large amounts of P4 supplied by the placenta (Mastorakos and Ilias, 2003). One
320 reason of decreased P4 in this study can be explained by a recent study that has reported

that PFOS exposure suppressed the secretion of P4 by inducing placental cell apoptosis (Zhang DY et al., 2015). However, p-for trend in quartile was not significant (Figure 1). Our result indicates the there is little dose-response toxicity of PFOS on P4. The inverse association between PFOS levels and the T/E2 ratio is consistent with the results from a Danish cross-sectional study of adult men (Joensen et al., 2013). Zhang Q et al. (2010) have reported that the T/E2 ratio in the seminal plasma of infertile men was significantly lower than that in normospermic men, which suggests that a lower T/E2 may indicate a corresponding reduction in Leydig cell function and spermatogenesis. In regard to steroid synthesis, the association of maternal PFOS with increased E2 and decreased T/E2 in this study is not consistent with the results in previous study that PFOS did not alter the aromatase activity (Kraugerud et al., 2011). It is also reported that the exposure to higher level PFOS seemed to inhibit the human 3beta-HSD and 17beta-HSD (Zhao B et al., 2010). Based on the decrease in P4 levels and unchanged T levels, our data indicate that PFOS exposure at low level had minimal effect on the pathway of steroid synthesis from P4 to T. Female P4 also showed the inverse association with maternal PFOS level in the present study. The decreased P4 in this study is consistent with the findings of Barrett et al. 2015, who reported that saliva PFOS levels were inversely associated with serum P4 levels among non-parous women

aged 25 to 35 with natural menstrual cycles (Barrett et al., 2015). There are few studies investigating the impact of PFASs on female progesterone, in spite of the great importance of progesterone for female reproductive health, including the pregnancy. More studies regarding the association between PFASs exposure and female's steroid hormones are needed to assess the importance of hormone levels in cord blood for reproductive health in later life.

We found that the male infants' levels of inhibin B were inversely associated with maternal PFOS levels in both our linear regression, and inverse tendency in quartile analyses. However, previous epidemiological studies among adults have reported no significant association between levels of PFASs and inhibin B (Joensen et al., 2009; Joensen et al., 2013; Vested et al., 2013). These results appear to suggest that the fetus' inhibin B secretion may be sensitive to PFAS exposure. One possible mechanism for PFOS-mediated decreases in inhibin B levels is the vacuolization of Sertoli cells in the seminiferous tubules and the blood-testis barrier disassembly in animals and in vitro tests (Qiu et al., 2013). In another study, higher PFOS exposure reduced the expression of inhibin subunit genes, such as *Inhba and Inhbb*, in male adult mice (Wan et al., 2011). These processes may explain how PFOS might inhibit the secretion of inhibin B by Sertoli cells. In contrast, we found that PFOA levels were

357 positively associated with the male infants' inhibin B levels in both our linear regression
358 analysis and quartile analysis. Thus, given that there is no definitive report regarding the
359 effects of PFOA on Sertoli cells or inhibin B levels, further studies are needed to
360 determine if there is a mechanism for PFOA-induced dysfunction. Inhibin B is produced
361 by Sertoli cells, and mainly down-regurates the FSH synthesis. Inhibin B is reported to
362 be associated with male reproductive health, such as testis volume and cryptorchidism
363 among infants (Main et al., 2006; Suomi et al., 2006). Therefore, the changes of inhibin
364 B level by PFOS and PFOA in this study indicate the disrupted reproductive function in
365 near future.

366 We observed that maternal PFOS levels were inversely associated with PRL
367 levels in female infants. Because there are no reports regarding the associations between
368 PFASs and PRL levels, the mechanism for PFOS or PFOA-mediated decreases in PRL
369 remains unknown.

370 The sex difference in the results is predictable due to the different hormone
371 levels and hormone function between males and females. Although the reason for sex
372 differences is not understood, one explanation for the sex difference is the faster
373 elimination time of PFASs in female compared to male in animal studies (Lau et al.,
374 2007; Zhang T et al., 2015).

In the multiple linear regression analysis, potential confounders were selected from Table 1 with $p<0.50$ and previous literatures. We conducted the backward procedure to exclude some confounders, and we did not find significant difference in the results in Table 4 and 5. In addition, hormone levels are controlled very strictly in the circulation by hypothalamic-pituitary-gonadal axis, hypothalamic-pituitary-adrenal axis. To maintain the hormone balances, the correlations between each hormone level are observed (Ex. T and E2; spearman's rho = 0.580). Moreover, hormones interact with each other, not only reproductive hormones but also other hormones. Although it is almost impossible to consider all correlations in the analyses, our results can demonstrate the disturbed balances of hormone levels by targeting different hormones in the negative-feedback- system and synthesis pathway.

A major strength of the present study is its prospective birth cohort design, which allowed us to estimate the effects of prenatal PFOS and PFOA exposure on fetal reproductive functions using prenatal and perinatal blood samples. However, there are several limitations in this study. Firstly, we included limited number of participants from original cohort because cord blood samples were only obtained from infants who were delivered via vaginal birth. We compared the maternal and infant characteristics of participants in this study with the original cohort population (n=514), as well as

393 participants with PFOS and PFOA data before delivery (n=323) (Supplemental Table 1).
394 The infants in our analyses had a higher gestational age and a heavier birth weight. The
395 median levels of PFOS and PFOA in maternal serum did not significantly differ
396 between the analyzed participants (PFOS: 5.2 ng/mL, PFOA: 1.4 ng/mL) and compared
397 groups, participants who had PFOS and PFOA data before their pregnancy (PFOS: 5.7
398 ng/mL, PFOA: 1.4 ng/mL) and the original cohort (PFOS: 5.2 ng/mL, PFOA: 1.3
399 ng/mL). However, it is possible that healthier children were included in our analyses,
400 which may have led us to underestimate the effects of PFOS and PFOA exposure.
401 Secondly, among the 257 mother-infant pairs with available maternal and cord blood
402 samples, we excluded 68 women whose blood samples were obtained after delivery
403 from our final analysis, considering the accurate reflection of prenatal exposure to
404 PFOS and PFOA exposure. However, that might decrease the statistical power of this
405 study. Furthermore, since we believe that the post-delivery samples also reflected those
406 women's PFAS levels during their pregnancy, as PFOS and PFOA have long half-lives
407 in human serum (5.4 years and 3.8 years, respectively), we performed the analyses
408 including those 68 mothers (Supplemental Table 2 to 5). The results did not
409 significantly affect our findings. Thirdly, we could not include all possible confounders
410 in regression analyses due to the lack of some information. We measured the levels of

411 reproductive hormones from cord blood in this study. It is important to note that
412 hormone levels dramatically change from the end of gestation to after birth (Kuijper et
413 al., 2013) and the levels of reproductive hormones measured in cord blood may be
414 affected by various factors like diurnal cyclicity, gestational week, duration of labor,
415 placenta weight, and the presence of pre-eclampsia (Hollier et al., 2014; Keelan et al.,
416 2012). Although we could use gestational age as a confounding factor and we also
417 checked the seasonal variation of hormone levels, and no significant difference was
418 found, our results in regression analyses might not reflect affected hormone levels
419 accurately. It is also noted that the results of multiple comparison should be carefully
420 considered because there is a 5% chance of incorrectly rejecting the null hypothesis
421 (Hubbard, 2011). However, we did not use a method to counteract the multiple
422 comparison error because it would increase the probability of false negative results.
423 In addition, simultaneous exposure to other chemical compounds that may
424 affect the levels of reproductive hormones should be considered. Our previous study
425 reported that maternal concentrations of PFOS and PFOA showed weak but
426 significantly correlated with polychlorinated biphenyls (PCBs), and mercury (Hg), due
427 to the similar exposure sources (Miyashita et al., 2015). As additional analyses, we also
428 added the levels of polychlorinated dibenzo-pdioxinsand -dibezofurans

(PCDDs/PCDFs) and polychlorinated biphenyls (PCBs) into final adjusted model in Table 4 and 5 as confounders, and the results were almost same as those in Table 4 and Table 5 in the directions and significance (data not shown). Therefore, we believe that our results indicated that PFOS and PFOA independently influenced the reproductive hormone levels in the different mechanisms from how PCDDs/PCDFs and PCBs did.

## 5. Conclusion

In summary, this study found that prenatal PFOS exposure significantly increased the male infant's level of E2 and decreased the male infants' levels of T/E2, P4 and inhibin B, and that PFOA associated their increased inhibin B levels. Similarly, PFOS was negatively associated with the female infants' P4 and PRL levels. These results suggest that in utero exposure to PFOS and PFOA, even at relatively low levels, has adverse effects on fetuses' synthesis of steroid hormones, their Leydig cell function, and their Sertoli cell function. However, our group has previously reported an increasing trend for PFAS levels with longer carbon chains (e.g., perfluorononanoic acid and perfluorodecanoic acid) instead of decreasing trend for PFOA and PFOA, in the plasma of pregnant women between 2003 and 2011 (Okada et al. 2013). Therefore, future studies are needed to verify the effects of PFASs with longer carbon chains on

fetal reproductive functions. These studies should also include long-term follow-up regarding the fetuses' reproductive development, in order to elucidate the outcomes of altered hormone levels at the fetal stage.

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678 **Figure Legends**

679

680

681 **Figure 1. Least square means of each reproductive hormone in cord blood among**

682 **male infants, according to maternal serum PFOS or PFOA concentration quartiles.**

683 (A) male E2 according to PFOS, (B) male T/E2 according to PFOS, (C) male P4

684 according to PFOS, (D) male inhibin B according to PFOS, (E) male INSL3 according

685 to PFOS, and (F) male inhibin B according to PFOA. PFOS and PFOA levels were

686 divided into 4 categories. PFOS: 1.5 ≦ Quartile (Q) 1 ≦ 3.9, 3.9 < Q2 ≦ 5.2, 5.2 <

687 Q3 ≦ 7.1, 7.1 < Q4 ≦16.2, PFOA : 0.25 ≦ Quartile (Q) 1 ≦ 0.9, 0.9 < Q2 ≦

688 1.4, 1.4 < Q3 ≦ 2.2, 2.2 < Q4 ≦5.3. Results are based on multiple linear regression

689 models that were adjusted for maternal factors (age, parity, body mass index before

690 pregnancy, annual income, smoking during pregnancy, caffeine consumption during

691 pregnancy, and gestational weeks of blood sampling for PFOS and PFOA

692 measurements) and infant factors (gestational age at birth). *p < 0.05, **p < 0.01

693 compared to the first quartile, as calculated using the Hsu-Dunnett method. LSMs are

694 indicated in rhombus and the black dots depict the upper and lower 95% CI. PFOS:

695 perfluorooctane sulfonate, PFOA: perfluorooctanoate.

696

**Figure 2. Least square means of each reproductive hormone in cord blood among female infants, according to maternal serum PFOS concentration quartiles.** (A) female P4 according to PFOS and (B) female PRL according to PFOS. PFOS levels were divided into 4 categories. PFOS: 1.5 ≦ Quartile (Q) 1 ≦ 3.9, 3.9 < Q2 ≦ 5.2, 5.2 < Q3 ≦ 7.1, 7.1 < Q4 ≦16.2. Results are based on multiple linear regression models that were adjusted for maternal factors (age, parity, body mass index before pregnancy, annual income, smoking during pregnancy, caffeine consumption during pregnancy, and gestational weeks of blood sampling for PFOS and PFOA measurements) and infant factors (gestational age at birth). *p < 0.05, **p < 0.01 compared to the first quartile, as calculated using the Hsu-Dunnett method. LSMs are indicated in rhombus and the black dots depict the upper and lower 95% CI. PFOS: perfluorooctane sulfonate.

**Table 1. Maternal perfluorooctane sulfonate and perfluorooctanoate concentrations according to maternal and infant characteristics. (n=189)**

| Characteristics | | N (%) | Mean ± SD | PFOS (ng/mL) | | PFOA (ng/mL) | |
|---|---|---|---|---|---|---|---|
| | | | | Median (IQR) | p-value | Median (IQR) | p-value |
| **Maternal characteristics** | | | | | | | |
| Age at delivery (years) | | 189 | 29.7 ± 4.8 | ρ = –0.060 | 0.483 | ρ = –0.049 | 0.500 |
| Pre-pregnancy BMI (kg/m$^2$) | | 189 | 21.2 ± 3.1 | ρ = –0.040 | 0.588 | ρ = –0.044 | 0.547 |
| Parity | Primiparous | 103 (54.5) | | 5.70 (4.20–8.00) | **0.001** | 1.70 (1.20–2.40) | **<0.001** |
| | Multiparous | 86 (45.5) | | 4.75 (3.10–6.28) | | 1.00 (0.70–1.53) | |
| Annual household income (million yen per year) | <5 | 132 (69.8) | | 5.20 (3.63–7.00) | 0.802 | 1.40 (0.90–2.18) | 0.277 |
| | ≥5 | 55 (30.2) | | 5.40 (4.20–7.50) | | 1.50 (0.90–2.20) | |
| Educational level (years) | ≤12 | 87 (46.0) | | 5.20 (4.00–6.80) | 0.773 | 1.30 (0.90–2.00) | 0.806 |
| | ≥13 | 102 (54.0) | | 5.35 (3.65–7.68) | | 1.50 (1.00–2.30) | |
| Fish consumption in a week (g/week) | | 189 | 47.2 ± 31.3 | ρ = –0.051 | 0.483 | ρ = –0.081 | 0.268 |
| Smoking during pregnancy | Nonsmoker | 148 (78.3) | | 5.30 (4.00–7.45) | **0.027** | 1.40 (0.90–2.20) | 0.065 |
| | Smoker | 41 (21.7) | | 4.70 (2.75–6.75) | | 1.20 (0.75–1.75) | |
| Alcohol consumption during | Nondrinker | 129 (68.3) | | 5.20 (3.95–7.40) | 0.300 | 1.40 (0.90–2.10) | 0.594 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| pregnancy | Drinker | 60 (31.7) | | 5.25 (3.63–7.08) | | 1.40 (0.90–2.28) | |
| Caffeine intake (mg/day) | | 189 | 142.3 ± 125.3 | ρ = –0.092 | 0.208 | ρ = –0.187 | **0.010** |
| Gestational week of blood sampling for PFOS and PFOA | | 189 | 33.3 ± 5.3 | ρ = –0.296 | **<0.001** | ρ = –0.194 | **0.008** |
| **Infant characteristics** | | | | | | | |
| Sex | Male | 83 (43.9) | | 5.40 (4.10–7.50) | 0.056 | 1.60 (1.00–2.40) | 0.055 |
| | Female | 106 (56.1) | | 5.15 (3.45–7.00) | | 1.35 (0.80–2.00) | |
| Birth weight (g) | Male | 83 (43.9) | 3168.4 ± 298.7 | ρ = –0.007 | 0.948 | ρ = –0.146 | 0.187 |
| | Female | 106 (56.1) | 3099.8 ± 350.0 | ρ = –0.211 | **0.030** | ρ = –0.162 | 0.097 |
| Gestational age (weeks) | | 189 | 39.5 ± 1.0 | ρ = 0.004 | 0.952 | ρ = 0.058 | 0.431 |

Statistical analyses were performed using Spearman's correlation test (ρ), or the Mann-Whitney U test and Kruskal-Wallis test, as appropriate. SD: standard deviation, PFOS: perfluorooctane sulfonate, PFOA: perfluorooctanoate, IQR: interquartile range, BMI: body mass index.

**Table 2. Distribution of reproductive hormone levels in cord blood**

| | Male (N = 83) | | | | Female (N = 106) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | n | Median | IQR | >LOD (%) | n | Median | IQR | >LOD (%) | p-value |
| Estradiol (ng/mL) | 83 | 5.37 | (3.64–7.80) | 100 | 106 | 5.01 | (3.48–6.67) | 100 | 0.259 |
| Testosterone (pg/mL) | 83 | 93.6 | (70.4–122.3) | 100 | 106 | 71.0 | (51.8–105.9) | 100 | **<0.001** |
| T/E2 | 83 | 17.2 | (12.0–22.6) | | 106 | 15.3 | (11.7–21.3) | | 0.273 |
| Progesterone (ng/mL) | 83 | 235.9 | (184.8–304.9) | 100 | 106 | 213.2 | (170.8–269.9) | 100 | 0.101 |
| LH (mIU/mL) | 80 | <LOD | (<LOD–0.83) | 32.5 | 104 | <LOD | (<LOD–<LOD) | 0.9 | **<0.001** |
| FSH (mIU/mL) | 80 | <LOD | (<LOD–0.65) | 43.4 | 103 | <LOD | (<LOD–<LOD) | 0.0 | **<0.001** |
| SHBG (nmol/L) | 83 | 16.3 | (13.5–19.0) | 100 | 106 | 15.6 | (13.2–18.5) | 100 | 0.382 |
| T/SHBG | 83 | 5.73 | (3.85–7.97) | | 106 | 4.38 | (3.31–6.39) | | 0.801 |
| PRL (ng/mL) | 80 | 84.7 | (67.1–114.8) | 100 | 105 | 86.9 | (56.8–122.5) | 99.1 | 0.693 |
| Inhibin B (pg/mL) | 83 | 43.2 | (32.6–56.8) | 100 | 106 | <LOD | (<LOD–11.7) | 23.6 | **<0.001** |
| INSL3 (ng/mL) | 80 | 0.28 | (0.24–0.32) | 100 | 20 | 0.20 | (0.17–0.25) | 100 | **<0.001** |

P-values were calculated using the Mann-Whitney U test. IQR: interquartile range, LOD: limit of detection, LH: luteinizing hormone, FSH: follicle stimulating hormone, SHBG: sex hormone-binding globulin, PRL: prolactin, INSL3: insulin-like 3.

**Table 3. Correlations between maternal perfluorooctane sulfonate and perfluorooctanoate levels and infant reproductive hormone levels**

| | Male (N = 83) | | | | Female (N = 106) | | | |
|---|---|---|---|---|---|---|---|---|
| | PFOS | | PFOA | | PFOS | | PFOA | |
| | Spearman's ρ | p-value | Spearman's ρ | p-value | Spearman's ρ | p-value | Spearman's ρ | p-value |
| Estradiol (ng/mL) | **0.253** | **0.021** | 0.085 | 0.444 | 0.084 | 0.391 | –0.033 | 0.739 |
| Testosterone (pg/mL) | –0.006 | 0.957 | –0.094 | 0.396 | 0.024 | 0.805 | –0.006 | 0.950 |
| T/E2 | **–0.330** | **0.002** | –0.206 | 0.062 | 0.005 | 0.955 | –0.042 | 0.668 |
| Progesterone (ng/mL) | –0.051 | 0.649 | **0.260** | **0.018** | –0.147 | 0.133 | 0.175 | 0.072 |
| LH (mIU/mL) | –0.081 | 0.475 | 0.027 | 0.815 | | n.d. | | n.d. |
| FSH (mIU/mL) | 0.003 | 0.982 | –0.122 | 0.280 | | n.d. | | n.d. |
| SHBG (nmol/L) | –0.038 | 0.733 | 0.127 | 0.253 | –0.125 | 0.201 | –0.062 | 0.527 |
| T/SHBG | 0.019 | 0.865 | –0.162 | 0.143 | 0.030 | 0.767 | 0.005 | 0.957 |
| PRL (ng/mL) | –0.113 | 0.318 | 0.151 | 0.182 | **–0.350** | **<0.001** | –0.095 | 0.333 |
| Inhibin B (pg/mL) | **–0.447** | **<0.001** | **0.230** | **0.037** | | n.d. | | n.d. |
| INSL3 (ng/mL) | –0.082 | 0.465 | 0.146 | 0.196 | | n.d. | | n.d. |

PFOS: perfluorooctane sulfonate, PFOA: perfluorooctanoate, n.d.: not determined, LH: luteinizing hormone, FSH: follicle stimulating hormone, SHBG: sex hormone-binding globulin, PRL: prolactin, INSL3: insulin-like 3.

**Table 4. Linear regression models of maternal perfluorooctane sulfonate and perfluorooctanoate levels and reproductive hormone levels among male infants (n=83)**

| | Male | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PFOS | | | | | | | | PFOA | | | | | | | |
| | Crude | | | | Adjusted | | | | Crude | | | | Adjusted | | | |
| | B | (95% CI) | | p-value | B | (95% CI) | | p-value | B | (95% CI) | | p-value | B | (95% CI) | | p-value |
| Estradiol (ng/mL) | **0.330** | **0.027** | **0.632** | **0.033** | **0.372** | **0.057** | **0.687** | **0.021** | 0.026 | –0.223 | 0.275 | 0.836 | –0.134 | –0.436 | 0.168 | 0.378 |
| Testosterone (pg/mL) | –0.096 | –0.412 | 0.220 | 0.548 | –0.027 | –0.367 | 0.312 | 0.872 | –0.206 | –0.455 | 0.042 | 0.103 | –0.160 | –0.474 | 0.154 | 0.313 |
| T/E2 | **–0.425** | **–0.679** | **0.171** | **0.001** | **–0.399** | **–0.643** | **–0.156** | **0.002** | **–0.232** | **–0.443** | **–0.022** | **0.031** | –0.041 | –0.224 | 0.143 | 0.662 |
| Progesterone (ng/mL) | **–0.365** | **–0.675** | **–0.056** | **0.021** | **–0.344** | **–0.678** | **–0.010** | **0.043** | 0.171 | –0.081 | 0.424 | 0.181 | 0.258 | –0.056 | 0.571 | 0.105 |
| LH (mIU/mL) | –0.132 | –0.518 | 0.253 | 0.497 | –0.243 | –0.643 | 0.158 | 0.231 | –0.085 | –0.229 | 0.399 | 0.592 | 0.071 | –0.301 | 0.443 | 0.704 |
| FSH (mIU/mL) | 0.036 | –0.247 | 0.319 | 0.802 | –0.027 | –0.321 | 0.267 | 0.854 | –0.121 | –0.349 | 0.108 | 0.297 | –0.141 | –0.410 | 0.128 | 0.300 |
| SHBG (nmol/L) | –0.044 | –0.160 | 0.072 | 0.455 | –0.051 | –0.167 | 0.063 | 0.374 | 0.052 | –0.041 | 0.144 | 0.270 | 0.009 | –0.0998 | 0.116 | 0.870 |
| T/SHBG | –0.052 | –0.377 | 0.273 | 0.751 | 0.024 | –0.318 | 0.366 | 0.890 | **–0.258** | **–0.511** | **–0.005** | **0.046** | –0.169 | –0.484 | 0.147 | 0.290 |
| PRL (ng/mL) | –0.102 | –0.297 | 0.094 | 0.303 | –0.132 | –0.341 | 0.077 | 0.212 | 0.099 | –0.059 | 0.258 | 0.217 | 0.043 | –0.152 | 0.237 | 0.664 |
| Inhibin B (pg/mL) | **–0.423** | **–0.598** | **–0.249** | **<0.001** | **–0.439** | **–0.620** | **–0.257** | **<0.001** | **0.182** | **0.029** | **0.335** | **0.021** | **0.197** | **0.009** | **0.384** | **0.040** |
| INSL3 (ng/mL) | **–0.159** | **–0.317** | **–0.002** | **0.047** | –0.139 | –0.303 | –0.025 | 0.095 | 0.054 | –0.077 | 0.184 | 0.418 | 0.121 | –0.030 | 0.273 | 0.114 |

Adjusted for maternal factors (age, parity, body mass index before pregnancy, annual income, smoking during pregnancy, caffeine consumption during pregnancy, and gestational weeks of blood sampling for PFOS/PFOA measurement) and infant factors (gestational age at birth). PFOS: perfluorooctane sulfonate, PFOA: perfluorooctanoate, LH: luteinizing hormone, FSH: follicle stimulating hormone, SHBG: sex hormone-binding globulin, PRL: prolactin, INSL3: insulin-like 3.

**Table 5. Linear regression models of maternal perfluorooctane sulfonate and perfluorooctanoate levels and reproductive hormone levels among female infants (n=106)**

| | Female | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PFOS | | | | | | | | PFOA | | | | | | | |
| | Crude | | | | Adjusted | | | | Crude | | | | Adjusted | | | |
| | B | (95% CI) | | p-value | B | (95% CI) | | p-value | B | (95% CI) | | p-value | B | (95% CI) | | p-value |
| Estradiol (ng/mL) | 0.136 | –0.086 | 0.357 | 0.227 | 0.081 | –0.148 | 0.312 | 0.481 | 0.033 | –0.107 | 0.173 | 0.638 | –0.040 | –0.194 | 0.114 | 0.606 |
| Testosterone (pg/mL) | 0.044 | –0.257 | 0.344 | 0.775 | 0.069 | –0.261 | 0.400 | 0.679 | –0.044 | –0.251 | 0.164 | 0.678 | –0.031 | –0.265 | 0.203 | 0.796 |
| T/E2 | –0.092 | –0.349 | 0.164 | 0.478 | –0.013 | –0.284 | 0.259 | 0.926 | –0.003 | –0.181 | 0.174 | 0.970 | 0.069 | –0.123 | 0.261 | 0.476 |
| Progesterone (ng/mL) | **–0.432** | **–0.766** | **–0.098** | **0.012** | **–0.552** | **–0.894** | **–0.210** | **0.002** | 0.106 | –0.131 | 0.344 | 0.377 | 0.039 | –0.216 | 0.293 | 0.764 |
| SHBG (nmol/L) | –0.165 | –0.386 | 0.057 | 0.143 | –0.180 | –0.417 | 0.057 | 0.135 | –0.103 | –0.256 | 0.050 | 0.185 | –0.117 | –0.286 | 0.051 | 0.169 |
| T/SHBG | 0.208 | –0.171 | 0.588 | 0.279 | 0.249 | –0.158 | 0.657 | 0.227 | 0.060 | –0.204 | 0.323 | 0.656 | 0.087 | –0.203 | 0.377 | 0.554 |
| PRL (ng/mL) | **–0.426** | **–0.677** | **–0.174** | **0.001** | **–0.491** | **–0.764** | **–0.218** | **0.001** | –0.112 | –0.294 | 0.071 | 0.229 | –0.157 | –0.361 | 0.047 | 0.131 |

Adjusted for maternal factors (age, parity, body mass index before pregnancy, annual income, smoking during pregnancy, caffeine consumption during pregnancy, and gestational weeks of blood sampling for PFOS/PFOA measurement) and infant factors (gestational age at birth). PFOS: perfluorooctane sulfonate, PFOA: perfluorooctanoate, SHBG: sex hormone-binding globulin, PRL: prolactin

**Figure(s)**

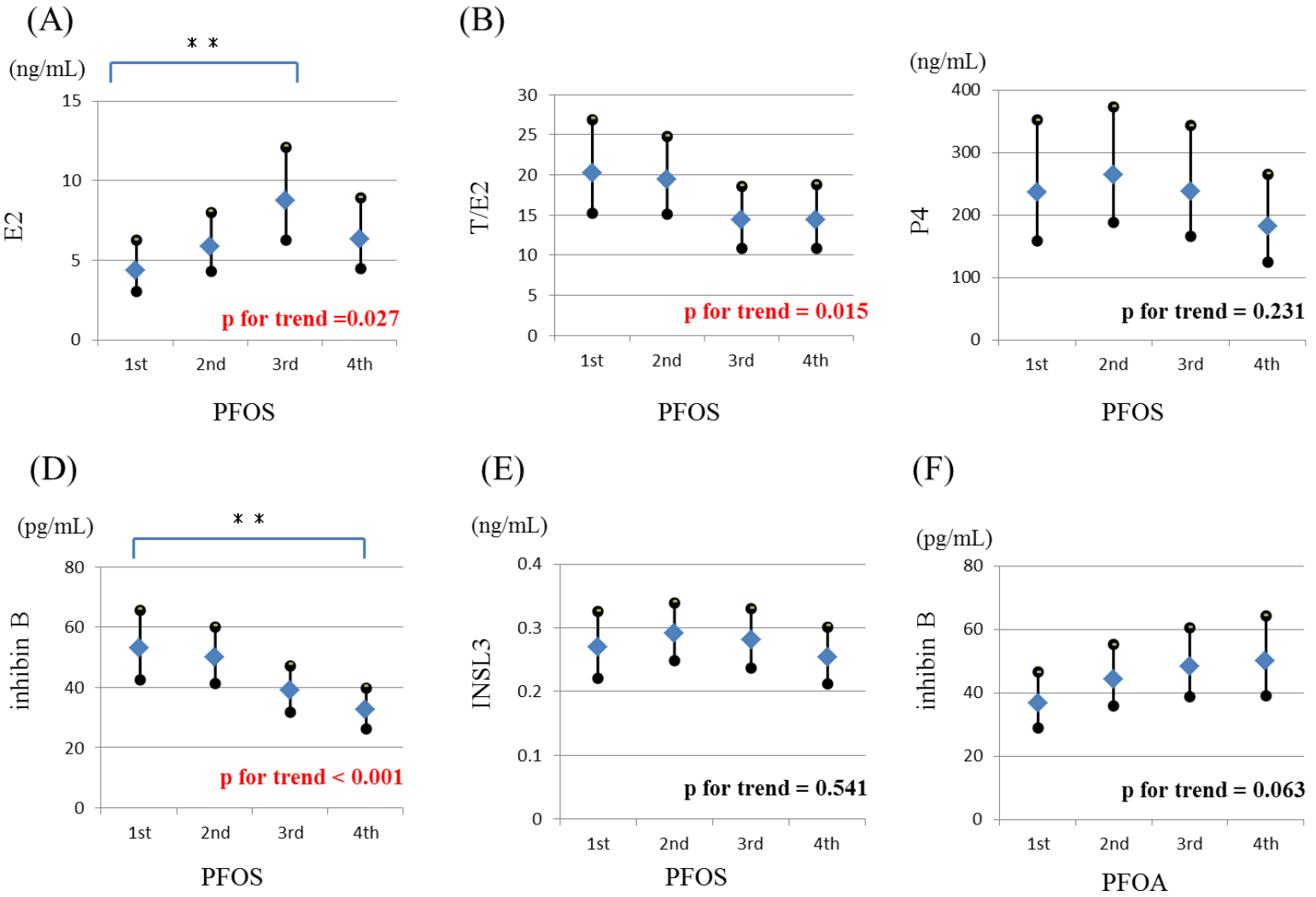


Figure 1.

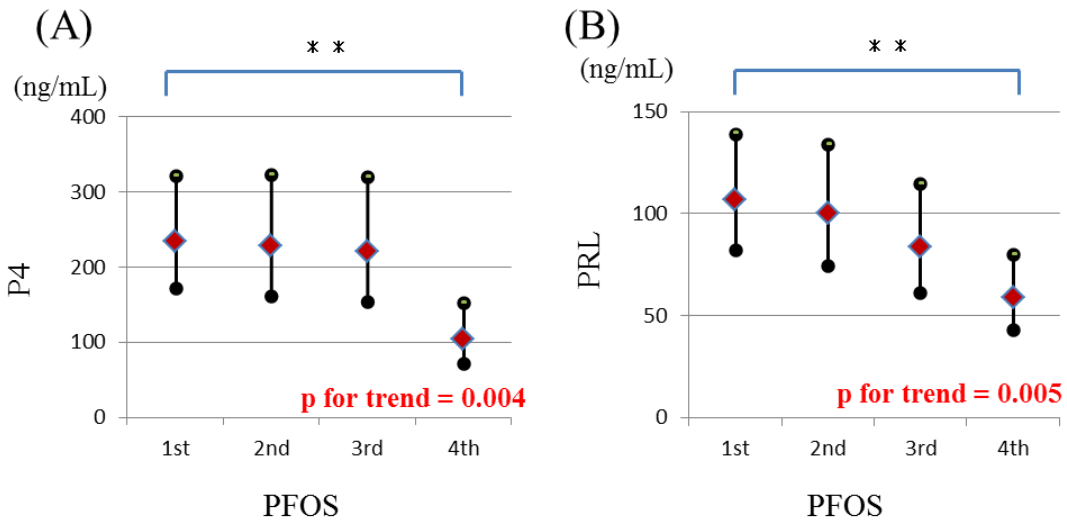
(A)
* *
(ng/mL)
400
300
200
100
0
P4
p for trend = 0.004
1st
2nd
3rd
4th
PFOS
(B)
* *
(ng/mL)
150
100
50
0
PRL
p for trend = 0.005
1st
2nd
3rd
4th
PFOS

Figure 2.