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Abstract: Polyfluoroalkyl substances (PFASs) are a group of common chemicals that ubiquitously exist in wildlife and humans. However, few studies have researched the effect of PFASs on reproductive hormones in adolescents. To provide information in this regard, we recruited 225 Taiwanese adolescents aged 13-15 years from 2009 to 2010 to investigate the relationship between serum PFASs (PFOS, PFOA, PFBS, PFDA, PFDoA, PFHxA, PFHxS, PFNA and PFTA) and reproductive hormone concentrations using a cross-sectional study design. Results showed PFOS and PFTA levels were highest among the PFASs, with a median concentrations of 29.9 (interquartile range: 13.0-43.8) ng/mL and 6.0 (0.6-25.9) ng/mL in males, and a median concentrations of 28.8 (14.8-42.6) ng/mL and 4.5 (0.3-18.4) ng/mL in females. After adjustment for confounding factors, nonsignificant associations between PFASs and reproductive hormone were found except for PFNA with ln(estradiol) ( $\beta=0.2060$ , 95%CI: 0.0016, 0.4105). When stratified by sex, more significant associations were found in males than in females. Among males, PFASs were negatively associated with ln(testosterone) level for PFOS ( $\beta=-0.0029$ , 95%CI: -0.0055, -0.0003), PFDA ( $\beta=-0.2565$ , 95%CI: -0.4135, -0.0994), PFHxA ( $\beta=-0.3095$ , 95%CI: -0.5942, -0.0248), and PFNA ( $\beta=-0.4233$ , 95%CI: -0.6998, -0.1467). Furthermore, male participant ln(estradiol) levels were positively associated with PFOA ( $\beta=0.0921$ , 95%CI: 0.0186, 0.1656), and PFHxS ( $\beta=0.0462$ , 95%CI: 0.0020, 0.0905). Among females, a significant relationship was found only for PFDoA with ln(testosterone) ( $\beta=-0.0119$ , 95%CI: -0.0227, -0.0010). In conclusion, this study showed higher levels of PFASs coincide with lower testosterone and higher estradiol levels, and more significant associations of PFASs with reproductive hormone were found in males than in females.

**Association of Perfluoroalkyl Substances Exposure with Reproductive Hormone Levels  
in Adolescents: by Sex Status**

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10

## Abstract

Polyfluoroalkyl substances (PFASs) are a group of common chemicals that ubiquitously exist in wildlife and humans. However, few studies have researched the effect of PFASs on reproductive hormones in adolescents. To provide information in this regard, we recruited 225 Taiwanese adolescents aged 13-15 years from 2009 to 2010 to investigate the relationship between serum PFASs (PFOS, PFOA, PFBS, PFDA, PFDoA, PFHxA, PFHxS, PFNA and PFTA) and reproductive hormone concentrations using a cross-sectional study design. Results showed PFOS and PFTA levels were highest among the PFASs, with a median concentrations of 29.9 (interquartile range: 13.0-43.8) ng/mL and 6.0 (0.6-25.9) ng/mL in males, and a median concentrations of 28.8 (14.8-42.6) ng/mL and 4.5 (0.3-18.4) ng/mL in females. After adjustment for confounding factors, nonsignificant associations between PFASs and reproductive hormone were found except for PFNA with  $\ln(\text{estradiol})$  ( $\beta=0.2060$ , 95%CI: 0.0016, 0.4105). When stratified by sex, more significant associations were found in males than in females. Among males, PFASs were negatively associated with  $\ln(\text{testosterone})$  level for PFOS ( $\beta=-0.0029$ , 95%CI: -0.0055, -0.0003), PFDA ( $\beta=-0.2565$ , 95%CI: -0.4135,-0.0994), PFHxA ( $\beta=-0.3095$ , 95%CI: -0.5942,-0.0248), and PFNA ( $\beta=-0.4233$ , 95%CI:-0.6998, -0.1467). Furthermore, male participant  $\ln(\text{estradiol})$  levels were positively associated with PFOA ( $\beta=0.0921$ , 95%CI: 0.0186, 0.1656), and PFHxS ( $\beta=0.0462$ , 95%CI: 0.0020, 0.0905). Among females, a significant relationship was found only for PFDoA with  $\ln(\text{testosterone})$  ( $\beta=-0.0119$ , 95%CI: -0.0227, -0.0010). In conclusion, this study showed higher levels of PFASs coincide with lower testosterone and higher estradiol levels, and more significant associations of PFASs with reproductive hormone were found in males than in females.

**Key words:** perfluoroalkyl substances; testosterone; estradiol; sex; adolescents

1    **Abbreviations**

- 2    PFASs: polyfluoroalkyl substances
- 3    PFBS: perfluorobutane sulfonate
- 4    PFHxS: perfluorohexane sulfonate
- 5    PFOS: perfluorooctane sulfonate
- 6    PFHxA: perfluorohexane acid
- 7    PFHpA: perfluoroheptanoic acid
- 8    PFNA: perfluorononanoic acid
- 9    PFOA: perfluorooctanoic acid
- 10   PFDA: perfluorodecanoic acid
- 11   PFDoA: perfluorododecanoic acid
- 12   PFTA: perfluorotetradecanoic acid
- 13   95%CI: 95% confidence intervals

## 1. Introduction

Reproductive hormones determine sex differences and control organ function and skeletal muscle growth. Testosterone and estradiol, which are steroid hormones, are primarily secreted from testes in males and ovaries in females (Cheng et al. 2010; Wall et al. 2014). Small amounts of both hormones are released by the adrenal glands in both sexes (Shea et al. 2014). During puberty, serum reproductive hormones rise dramatically and insufficient levels will induce adverse health conditions, such as infertility (Patel et al. 2015), loss of bone density (Morley et al. 1997; Schow et al. 1997), obesity (Pinola et al. 2012), and depression (Barrett-Connor et al. 1999).

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) are a large group of synthetic chemicals which are widely used in various manufacturing and industrial processes (Rahman et al. 2014). The combination of carbon and fluorine atoms in the aliphatic carbon backbone of these substances, in which fluorine has replaced hydrogen atoms, allows PFASs to resist degradation and makes them extremely persistent in environmental and in biological samples (Lindstrom et al. 2011). The potential harmful effects of PFASs to human health have raised concern. Until now, most PFASs toxicity studies have investigated developmental deficits (Lau et al. 2004), neurotoxicity (Mariussen 2012), and immune system function (Grandjean et al. 2012).

Several studies have found that the PFAS affect the endocrine system in vivo and in vitro (Jensen et al. 2008). Animal studies have demonstrated that a higher exposure to PFOA

1 concentrations is associated with lower testosterone levels and higher estradiol levels in male  
2 adult rats (Biegel et al. 1995). For PFOS, higher exposure was associated with lower estradiol  
3 levels in male monkeys (Seacat et al. 2002) and decreased testosterone levels in male fish  
4 (Arukwe et al. 2013). Furthermore, PFNA is associated with a dramatic decrease in  
5 testosterone levels and an elevation in estradiol levels in rats (Feng et al. 2009).

6  
7 Epidemiological evidence is limited and not consistent. In a study of 2,292 children aged 6-9  
8 years old, Lopez-Espinosa et al. (2016) reported significant associations of reproductive  
9 hormone levels with certain PFASs. The study demonstrated there were significant inverse  
10 associations of testosterone levels with PFOA and PFOS in boys, and PFOS in girls, as well  
11 as significant inverse associations of estradiol levels with PFOS concentration in girls. The  
12 same author previously reported delayed puberty in children is correlated with PFOS and  
13 PFOA levels (Lopez-Espinosa et al. 2011). Additionally, a negative association between  
14 serum PFOS concentration and testosterone levels in young Danish men (median age 19  
15 years, n=247) was reported by Joensen et al. (2013). In a separate study 25,957 adult women  
16 in the same Danish community, PFOS and PFOA concentrations were positively associated  
17 with earlier menopause, and PFOS concentrations were associated with lower estradiol levels  
18 (Knox et al. 2011). However, no association was found between PFOA or PFOS  
19 concentrations and testosterone and estradiol levels in Danish men (median age, 19 years)  
20 (Joensen et al. 2009). Olsen et al. (1998) found no association between PFOA or PFOS  
21 concentrations and testosterone and estradiol levels in American workers.



1 Studies investigating the impact of PFASs on human reproductive health are limited and  
2 controversial, especially in adolescents. Thus, the aim of the present study was to assess the  
3 associations between PFASs concentrations and the levels of reproductive hormones in  
4 adolescents aged 13-15.

## **2. Methods**

### **2.1 Study participants**

The study participants were from the entire control cohort of the Genetics and Biomarkers study for Childhood Asthma (GBCA) in Taiwan. This sample of 225 healthy adolescents (102 boys and 124 girls aged 13-15 years during 2009-2010), was selected from seven public schools in the Taipei city of Northern Taiwan (Zeng et al. 2015). Each school contributed a population of children who had no personal or family history of asthma. The response rate was 72%, and the age of participants ranged from 11.9 to 15.1 years (mean  $\pm$  SD, 13.6  $\pm$  0.7 years). After receiving written informed consent from the adolescents and their parents, the participants were surveyed to collect information concerning demographics and environmental exposures. Information regarding the smoking status and smoking history of each participant's adult household members and adult visitors was collected. Serum samples were collected for each child after 8 hours of fasting. A trained technician measured the height, weight, waist circumference and blood pressure of each child. The study protocol was approved by the Institutional Review Board of the National Taiwan University Hospital Research Ethics Committee and was in compliance with the principles outlined in the Helsinki Declaration (Declaration of Helsinki 1990).

### **2.2 Covariates**

Information regarding demographic characteristics such as age, sex, parental education, environmental tobacco smoke (ETS) exposure and exercise were collected via a self-reported questionnaire. ETS information was collected from the current and past household smoking

status of each participant's adult household members and regular household visitors. Regular exercise was defined as 'yes' if the participant has exercised at least 1 h per day in the past year excluding physical education in the school, and 'no' if they have not. Trained study staff recorded the weight and height of the participants and calculated the body mass index (BMI; weight in kg per height in m<sup>2</sup>).

### **2.3 Serum reproductive hormone determination and serum PFAS measurements**

Clinical laboratory tests were performed at an accredited clinical diagnostic laboratory. The primary outcome of interest was serum reproductive hormone levels including testosterone and estradiol. Serum was extracted from red blood cells, stored in tubes, and chilled prior to being shipped to an analytical laboratory. We measured reproductive hormones in serum by immunoluminometric assay with an Architect random access assay system (Abbott Diagnostics, AbbottPark, IL). The limit of detection (LOD) for estradiol and testosterone were 18 pmol/L and 0.23 nmol/L, respectively. Volumes used for analyses were 50 µL for testosterone and estradiol. All the measurements were duplicated and the average of the two values was calculated and used as the concentrations of each sample. The intra-assay coefficients of variation of these measurements were all below 10%, and the inter-assay coefficients of variation were all below 15%. A total of 67% of the blood samples were drawn before 12:00 hours, and 33% between 12:00-17:00 hours.

PFASs were measured from 0.5 mL of serum using Agilent high-performance liquid chromatography (HPLC) in tandem with an Agilent 6410 Triple Quadrupole (QQQ) mass

spectrometer (MS/MS) (Agilent, Palo Alto and Santa Clara, CA). Detailed information about standards and reagents, sample preparation and extraction, instrumental analysis, quality assurance and quality control, and recovery experiments in the present study is provided in Supplementary Material and is described elsewhere (Hansen et al. 2001). Ten PFASs were analyzed in serum samples: perfluorobutanesulfonate (PFBS), perfluorohexanesulfonate (PFHxS), PFOS, perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluorododecanoic acid (PFDoA), and perfluorotetradecanoic acid (PFTA). The limit of quantification (LOQ) for PFOS, PFOA and PFNA was 0.03ng/mL, for PFBS and PFHxS was 0.07 ng/mL, for PFDA and PFDoA was 0.1 ng/mL, for PFHpA and PFHxA was 0.05 ng/mL, and for PFTA was 0.02 ng/mL. All the measurements were duplicated and the average of the two values was calculated and used as the concentrations of each sample. For statistical tests, concentrations of PFASs below the LOQ were assigned a value equal to the LOQ divided by the square root of 2. Nearly half serum PFHpA levels were below the LOQ, therefore PFHpA was not included in the present statistical analyses.

## **2.4 Statistical analysis**

Statistical analyses were performed using SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA). Data were tested for normality (Q-Q plots) and homogeneity (Bartlett's test for unequal variances). Appropriate transformations were made as needed. Continuous variables with acceptable normality and homogeneity were given as the mean  $\pm$  SD, otherwise, they

were given as median and quartile 1(Q1)-quartile 3(Q3). The *t*-test was used to compare the normal distributed continuous variables between sex. Because PFAS concentrations were highly skewed, we utilized the Wilcoxon rank-sum test to compare PFAS concentrations between sex. Associations between categorical variables were tested by use of contingency tables and the Chi-square test. Multiple general linear models were used to estimate the association of reproductive hormone levels with a single PFASs exposure variable adjusted for identified covariates. Analyses were sex-stratified, but we also investigated differences by sex including the interaction of this variable with the contaminants in the joint models (i.e., models including girls and boys) (Lopez-Espinosa et al. 2016). Also, we conducted another multiple general linear model analysis to estimate the association of reproductive hormone levels with PFASs quartiles, using the lowest PFASs quartile as a reference group, and adjusting for identified covariates. We modeled an ordinal variable assigned to the median value for each corresponding quartile to identify trends in *p*-values. To determine the magnitude of other potential confounding, the following variables were examined using a backward deletion strategy: age (years), sex (boys vs. girls), parental education (<high school vs. ≥high school), body mass index (BMI, kg/m<sup>2</sup>), environmental tobacco smoke (ETS) exposure (yes vs. no), regular exercise (yes vs. no), and month of survey (November-December vs. July-September). If the estimated PFASs association changed by at least 10% after a covariate was added to the base model, the covariate was included in the final model. Correlations between PFASs and reproductive hormone were assessed via Spearman rank correlation tests. Results were considered statistically significant if they had a *p*-value of <0.05.

### 3. Results

The basic characteristics of the study cohort are shown in Table 1. The mean age of all participants (n=225) was  $13.6 \pm 0.7$  years; there was no significant differences by child sex ( $p=0.863$ ). Most PFASs were detected in over 94% of participant serum samples, except for PFDoA (84%) and PFHpA (53.3%). Because of the large number of samples below the LOQ, we did not conduct further analyses of PFHpA. There were large differences in the levels of all types of PFASs serum concentrations in the study sample with the highest concentration in PFOS. Our results showed that PFOS and PFTA levels were highest among the PFASs, with a median concentrations of 29.9 (interquartile range: 13.0-43.8) ng/mL and 6.0 (0.6-25.9) ng/mL in boys, and a median concentrations of 28.8 (14.8-42.6) ng/mL and 4.5 (0.3-18.4) ng/mL in girls. Median serum PFOS concentration in adolescents of 28.9 ng/mL was over 140 times the lowest concentration of PFHxA (median=0.2 ng/mL). The serum PFAS concentrations of boys and girls showed no significant difference. However, the reproductive hormone (testosterone and estradiol) levels were significantly different by child sex ( $p<0.001$ ). The levels of testosterone were higher in boys and the levels of estradiol were higher in girls. Compared with the results from other studies, the levels of testosterone and estradiol of the participating adolescents were within the normal range [see Supplemental Material, Table S1].

Additionally, the correlations between PFAS pollutants are presented in the Supplementary Material Table S2. Overall, many significant correlation coefficients of various PFAS pollutants were found, ranging from 0.139 for PFHxS and PFTA to 0.794 for PFDA and PFNA. Furthermore, the relationships of PFASs levels with reproductive hormones were also

evaluated (see Supplemental Material, Table S3). The results showed that significant negativity associations were found for testosterone with PFDA ( $\gamma_{\text{spearman}}=-0.138$ ,  $p=0.039$ ), and PFNA ( $\gamma_{\text{spearman}}=-0.145$ ,  $p=0.030$ ).

The results of the associations between PFASs concentrations and reproductive hormone levels are shown in Table 2 and Table 3. Level of testosterone was negatively associated with PFOS, PFDA, PFHxA, and PFNA levels among boys. As shown in Table 2, for a 1ng/mL increase in these types of PFASs, the male mean total  $\ln(\text{testosterone})$  levels were 0.0029 (95%CI: -0.0055, -0.0003) nmol/L, 0.2565 (95%CI: -0.4135, -0.0994) nmol/L, 0.3095 (95%CI: -0.5942, -0.0248) nmol/L, and 0.4233 (95%CI: -0.6998, -0.1467) nmol/L lower, respectively. Six of the nine PFASs were also negatively associated with testosterone levels in girls, but this was not statistically significant except for the PFDoA ( $\beta=-0.0119$ ; 95%CI: -0.0227, -0.0010). Male estradiol level, however, was positively associated with PFOA and PFHxS. When the serum concentrations of these PFASs were elevated by 1 ng/mL, mean  $\ln(\text{estradiol})$  levels increased by 0.0921 (95%CI: 0.0186, 0.1656) pmol/L, and 0.0462 (95%CI: 0.0020, 0.0905) pmol/L, respectively. A margin significant association was also found between PFNA and  $\ln(\text{estradiol})$  ( $\beta=0.3204$ ; 95CI: -0.0115, 0.6522,  $p=0.058$ ). All tested PFASs, except for PFHxA, were negatively, but not significantly, associated with estradiol levels in females (Table 3).

Interaction between sex and PFASs were estimated among adolescents. After adjustment for other confounding factors, we found a marginal significant interaction ( $P<0.15$ ) for testosterone between sex and PFOS ( $P_{\text{interaction}}=0.060$ ) and PFNA ( $P_{\text{interaction}}=0.042$ ). As for estradiol, we did not find any significant interactions between sex and PFASs.

1 When boy and girl data were pooled together, none of the PFASs were significantly  
2 associated with serum levels of estradiol among the participants except for PFNA with  
3  $\ln(\text{estradiol})$  ( $\beta=0.2060$ , 95%CI: 0.0016, 0.4105). Furthermore, a negative trend for  
4 association with testosterone concentrations was significant for PFOS (Figure 1).

5



## Discussion

In the present study, we found that higher serum levels of PFASs were associated with lower testosterone and higher estradiol concentrations in adolescents, especially in C6-C9. Moreover, the reproductive hormone levels of males were significantly influenced by PFASs concentrations.

Our results show that PFOS and PFTA levels represented the highest concentrations of PFASs, with a median of 29.9 ng/mL and 6.0 ng/mL in boys, and a median of 28.8 ng/mL and 4.5 ng/mL in girls. The median concentrations of PFOS in adolescents in this study are much higher than average concentrations reported in American children aged 0-12 (Schechter et al. 2012) and in Australian children in age group 5-15 years (Toms et al. 2014) in 2010-11, while similar to the same age group in Australia in 2002-03 (Toms et al. 2014). Serum PFAS concentrations increased by three times in children living in the Taipei area from 2006-08 (Lin et al. 2011) to 2009-10 (Bao et al. 2014), while globally, serum PFAS concentrations declined (Fitz-Simon et al. 2013; Toms et al. 2014). For PFOA, our result is much lower than those reported by (Fitz-Simon et al. 2013; Toms et al. 2014). These results suggest that the main environmental contaminant in Taipei adolescents aged 13-15 years may be PFOS and not PFOA.

The present study shows that the concentration of PFASs in male and female adolescents is not statistically different. These findings are similar to those of Schechter et al. (2012) who analyzed 300 serum samples of American children from birth to 13 years of age, and found

1 that concentrations of PFOS, PFOA, PFNA, and PFHxS were not significantly different by  
2 sex. Results from a study in Australia including 2,420 donors aged 0-60 years in 2006–07  
3 also showed no apparent sex differences among children younger than 12 years of age (Toms  
4 et al. 2009). However, these results conflict with several studies reporting serum  
5 concentrations of PFASs to be higher in males than females. For example, Inuit children in  
6 Nunavik, Canada, were shown to have concentrations of PFASs significantly higher in the  
7 serum of male compared to that of their female counterparts. The median of PFOS and PFOA  
8 were 44.9 ng/L and 4.5 ng/L in males, and 20.6 ng/L and 1.8 ng/L in females, respectively  
9 (Turgeon O'Brien et al. 2012). The median of PFOS and PFOA were 74.5 ng/mL and 7.2  
10 ng/mL in men, and 28.5 ng/mL and 2.57 ng/mL in women, respectively among Greenlandic  
11 Inuits living in Nuuk with a median age of 50 years (Long et al. 2012). Similar results, at a  
12 much lower concentration level of PFASs, were found in Ukraine (Góralczyk et al. 2015).  
13 These results suggest that sex differences in PFASs concentrations occur with age. A possible  
14 reason may be the high PFASs time-dependent bioaccumulation, which is consistent with the  
15 definition of persistent organic pollutants. Another reason may involve menstruation;  
16 research has shown that menstruation enhances elimination of PFOS, leading to the lower  
17 PFOS concentration in women (Wong et al. 2014).

18  
19 Previous studies about the relationship between the PFASs concentrations and hormone  
20 levels in humans were limited and had inconsistent results [see Supplemental Material, Table  
21 S4]. Our results demonstrate that higher selected PFASs concentrations decrease the  
22 testosterone level and increase the estradiol level, and these associations were more apparent

1 in males than in females. These results are similar to other recent findings (Joensen et al.  
2 2009, 2013; Lopez-Espinosa et al. 2016, 2011). A recent cross-sectional analysis within the  
3 C8 Health Project evaluated the relationship between levels of PFASs and testosterone and  
4 estradiol in 2,292 children aged 6-9 years living near a chemical plant in the Mid-Ohio Valley  
5 (USA) with local contamination from PFOA. The results showed that PFOS concentrations  
6 (median: 22 ng/mL for boys, and 21 ng/mL for girls) were significantly associated with  
7 testosterone levels in boys (-5.8%, 95%CI: -9.4, -2.0%) and in girls (-6.6%, 95%CI: -10.1, -  
8 2.8%), and a significant delay of age pubertal development (defined by testosterone levels >  
9 50 ng/dL) was also found for PFOS in boys of the same population (Lopez-Espinosa et al.  
10 2011). Joensen et al. (2009) conducted a study in 105 Danish men (median age=19 years)  
11 during 2008–09 and reported that PFOS levels (median: 24.5 ng/mL) were negatively  
12 associated with testosterone ( $\beta$ =-0.087, 95%CI: -0.32, 0.15), although the association was not  
13 significant. However, when authors enlarged the study sample, they found that PFOS levels  
14 (median: 7.79 ng/mL) were significantly associated with lower testosterone ( $\beta$ =-0.010,  
15 95%CI:-0.020, -0.000) in 247 Danish men (Joensen et al. 2013). However, among Danish  
16 women, nonsignificant associations of these hormones with in utero PFOS concentration  
17 (median: 21.1 ng/mL) were found in a Danish population-based cohort study (Kristensen et al.  
18 2013). In contrast, results from the Avon Longitudinal Study of Parents and Children  
19 (ALSPAC) reported a positive relationship between total testosterone concentrations with  
20 prenatal concentrations of PFOS (median: 19.2 ng/mL) ( $\beta$ =0.18, 95%CI: 0.01, 0.35), PFOA  
21 (median: 3.6 ng/mL) ( $\beta$ =0.24, 95%CI: 0.05, 0.43), and PFHxS (median: 1.6 ng/mL) ( $\beta$ =0.18,  
22 95%CI: 0.00, 0.35) in 72 females aged 15 years (Maisonet et al. 2015). Tsai's et al. (2015)

recruited 540 Taiwanese participants aged 12-30 years from the same area as our study to investigate the association of PFOS (geometric mean  $\pm$  geometric standard deviation:  $7.78 \pm 2.40$  ng/mL) and PFOA ( $2.74 \pm 2.95$  ng/mL) with reproductive hormones. They found that, in the 12–17 year-old group, serum testosterone level showed a significant, negative association with different percentile categories of PFOS only in females, but not in males. They also found that higher PFOA concentrations related to lower serum levels of sex hormone-binding globulin only in females. Knox et al. (2011) studied 25,957 women aged 18–65 years in Americans and also found higher PFOS concentrations (median: 15.0-21.5 ng/mL) to be associated with lower estradiol levels ( $\beta = -3.65$ ,  $P < 0.001$ ). Furthermore, Olsen et al. (1988) reported PFOA was not significantly associated with testosterone or estradiol in American workers. Results from a pregnancy cohort established in Aarhus, Denmark in 1988-1989 showed nonsignificant relationship between PFOS and reproductive hormones in 169 male offspring (19-21 years of age) (Vested et al. 2013). Raymer et al. (2012) studied 256 American men during 2002-05 and found testosterone and estradiol levels were independent of PFOA (median: 9.2 ng/mL) or PFOS (median: 32.3 ng/mL) concentrations. Further studies will need to be undertaken to clarify the biological mechanisms underlying these observations.

The underlying mechanism on how PFASs compounds affect the endocrine system still remains unclear. Several hypotheses have been proposed including that PFASs exposure may affect synthesis of enzymes and steroidogenic, combine with estrogen receptors easily, and disrupt the reproductive axis (Biegel et al. 1995; López-Doval et al. 2015; Rosenmai et al. 2013). Biegel et al. (1995) clarified that exposure to PFOA elevated serum estradiol levels

1 due to aromatase induction and also inhibited testosterone release from Leydig cells (Biegel  
2 et al. 1995). Another study showed that PFOA and fluorotelomer alcohol exposure could  
3 produce estrogenic activities, and a combination exposure of 17beta-estradiol and PFASs  
4 revealed anti-estrogenic effects in hepatocytes of male tilapia (Liu et al. 2007). The authors  
5 proposed the estrogenic effect of PFASs may be mediated by the estrogen receptor pathway.  
6 Rosenmai et al. (2013) reported lower androgen and higher estrogen levels due to the effect  
7 of the steroidogenic pathway after exposure to fluorochemicals in vitro. In a recent study by  
8 López-Doval et al. (2015) of male rats, the authors found PFOS exposure induced diminution  
9 of the serum testosterone and estradiol levels due to the serotonin and neuropeptide Y activity  
10 decreasing on the reproductive axis.

11  
12 The mechanisms underlying the sex difference in response to PFASs exposure is unclear.  
13 Increasing experimental evidence has shown there are sex differences in excretion of PFAS  
14 compounds (Lau et al., 2007; Kudo et al., 2002). Kudo et al., (2002) reported the biological  
15 half-life of PFOA in male rats was 70-times longer than in female rats; the difference was  
16 mainly attributable to a difference in renal clearance (CL(R)). These researchers further  
17 pointed out that sex hormones might play an important role in the sex difference underlying  
18 PFOA CL(R). For example, castration of male rats resulted in a 14-fold increase in the PFOA  
19 CL(R) that, in turn, made it comparable to that of female rats. However, treatment with  
20 testosterone reduced the elevated PFOA CL(R) in castrated males. In the present study,  
21 although the serum concentrations of PFAS were a little higher in male than in female, the  
22 differences were not notable. Thus, this indicated to us that differences in elimination rates

1 between sexes in the present study might not be a basis for the other altered responses noted  
2 herein. We also compared the reproductive hormones levels by menarche status. However,  
3 only 8% females (n=19) have reported without menarche, and our population did not exhibit  
4 variations in their estradiol levels due to menarche ( $157.6 \pm 35.4$  pmol/L vs.  $150.9 \pm 67.2$   
5 pmol/L), which is the most important limitation that may have influenced our results. As for  
6 menstruation status in females with menarche, we did not collect such information during the  
7 sampling time. The difference of health response to PFASs between males and females  
8 remains unclear and needs to be explored further. Careful consideration of sex effects and  
9 exploration of nascent methods for quantitative sex analysis may help to elucidate sources of  
10 difference.

11  
12 One interesting finding shows that those PFASs with 6-10 perfluorinated carbons (PFOS,  
13 PFOA, PFDA, PFHxS, PFNA) significantly affect the reproductive hormone levels, even at  
14 low levels, while other PFASs did not show any obvious effects. This indicates that PFASs  
15 with between 6 and 10 perfluorinated carbons are more likely to affect the endocrine system.  
16 Our results are consistent with the results of other studies (Kjeldsen et al. 2013; Zeng et al.  
17 2015; Ng et al. 2014). Kjeldsen et al. (2013), for example, reported that PFHxS, PFOS and  
18 PFOA significantly increased estrogen receptor transactivity (ER), whereas PFHxS, PFOS,  
19 PFOA, PFNA and PFDA significantly antagonized androgen receptor activity (AR). The  
20 authors concluded that the five PFASs had potential interference effects of the ER or AR  
21 function. In a previous study we also showed that PFOS, PFOA and PFNA were positively  
22 associated with total cholesterol, low-density lipoprotein and triglycerides (Zeng et al. 2015).

1 In addition, a systematic review (Ng et al. 2014) indicated that PFASs and the serum albumin  
2 bind affinitively, which appears to reach its peak between 6 and 10 perfluorinated carbons.  
3 This suggests that PFASs with C6-C10 are difficult to excrete and their accumulation may  
4 influence of the endocrine system later in the development. Thus, future research may focus  
5 on the toxicity of special perfluourinated carbons of PFASs.

6  
7 The strength of this study is the research into a complete set of PFASs exposure and their  
8 influence on reproductive hormones. Moreover, the study clearly demonstrated changes in  
9 reproductive hormone levels in pubescent adolescents, which may have a profound influence  
10 in the development of their reproductive functions. However, we should also carefully  
11 interpret these results. First, we should be concerned about the possible effect of smaller  
12 sample size on results. As the results shown in Table 1, the sample size of participants was  
13 only 225. As known, increasing sample size is often the easiest way to boost the statistical  
14 power of a test, so, future studies will need to be undertaken to verify this point. Second,  
15 serum sex steroid concentrations may have variable diurnal cyclicality, and interpretation of  
16 levels is challenging (and sometimes controversial) even without trying to elucidate their  
17 relationship with PFAS. Therefore, with the childhood hormone changes in mind, the results  
18 of present study might be not totally comparable with results in adults.

19 The limitations of these analyses should be noted. Firstly, due to the cross-sectional study  
20 design, establishing the causal relationship between PFASs and reproductive hormones is not  
21 possible. Secondly, menarche or other puberty indicators were not taken into consideration.  
22 Adult studies have reported menarche status may influence serum PFAS concentrations and

reproductive hormones in females (Wong et al. 2014). Furthermore, as diurnal cyclicity impacts on sex steroid hormones variation were not confirmed through collection of additional data such as the time of the day of sample collection (Ankarberg and Norjavaara 1999; Ankarberg-Lindgren and Norjavaara 2004), future studies will need to be undertaken to verify this point. Finally, the associations of reproductive hormone levels and PFASs may be biased due to confounding correlations with other environmental pollutants.

In conclusion, we found that those PFASs with 6-10 perfluorinated carbons (PFOS, PFOA, PFDA, PFHxS and PFNA), significantly influenced reproductive hormone levels male adolescents. These higher PFASs levels were associated with lower testosterone and higher estradiol levels in 13-15 year old boys. Long-term cohort research is needed to further clarify these associations.

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## **Conflict of interest**

The authors do not have any conflict of interest to declare.



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1 **Table 1** Characteristics of Taiwanese adolescents in the study population

Characteristic	Total (n=225)	Boys (n=102)	Girls (n=123)	p-value <sup>#</sup>
Age(years)(median)	13.6±0.7(13.6)	13.6±0.7(13.6)	13.6±0.8(13.7)	0.863
Height(cm)(median)	159.8±7.0(159.0)	163.1±7.2(163.5)	157.1±5.5(157.0)	<0.001
Weight(kg)(median)	52.5±13.2(49.7)	55.9±15.4(51.4)	49.6±10.3(48.8)	<0.001
BMI(kg/m <sup>2</sup> )(median)	20.4±4.1(19.4)	20.8±4.7(19.5)	20.0±3.6(19.4)	0.139
Regular exercise <sup>*</sup>				
No	53(23.6)	17(16.7)	36(29.3)	0.027
Yes	172(76.4)	85(83.3)	87(70.7)	
Parental education <sup>*</sup>				
<High school	86(38.2)	42(41.2)	44(35.8)	0.406
≥High school	139(61.8)	60(58.8)	79(64.2)	
Environmental tobacco smoke (ETS) exposure <sup>*</sup>				
No	93(41.3)	42(41.2)	51(41.5)	0.965
Yes	132(58.7)	60(58.8)	72(58.5)	
Month of survey <sup>*</sup>				
July-September	156 (69.3)	72(70.6)	84(68.3)	0.710
November-December	69 (30.7)	30(29.4)	39(31.7)	
PFOS (ng/mL) <sup>†</sup>	28.9(14.1-43.0)	29.9(13.0-43.8)	28.8(14.8-42.6)	0.619
PFOA (ng/mL) <sup>†</sup>	0.5(0.4-1.3)	0.5(0.4-1.4)	0.5(0.4-1.2)	0.247
PFBS (ng/mL) <sup>†</sup>	0.5(0.4-0.5)	0.5(0.4-0.5)	0.5(0.4-0.5)	0.469
PFDA (ng/mL) <sup>†</sup>	0.9(0.8-1.2)	0.9(0.8-1.1)	1.0(0.8-1.2)	0.394
PFDOA (ng/mL) <sup>†</sup>	2.7(0.8-6.0)	2.4(0.7-5.9)	3.1(0.9-6.2)	0.894
PFHxA (ng/mL) <sup>†</sup>	0.2(0.1-0.3)	0.2(0.1-0.3)	0.2(0.1-0.3)	0.911
PFHxS (ng/mL) <sup>†</sup>	1.3(0.6-2.8)	1.4(0.7-2.6)	1.2(0.5-3.0)	0.792
PFNA (ng/mL) <sup>†</sup>	0.8(0.6-1.1)	0.8(0.6-1.0)	0.9(0.6-1.1)	0.232
PFTA (ng/mL) <sup>†</sup>	5.0(0.3-23.3)	6.0(0.6-25.9)	4.5(0.3-18.4)	0.763
Testosterone(nmol/l)(median)	9.2±8.9(9.2)	17.6±6.9(17.2)	2.2±0.6(2.1)	<0.001
Estradiol(pmol/l)(median)	151.5±65.0(140.6)	116.9±48.9(111.0)	180.1±62.9(174.3)	<0.001

2 Values of normal distributed continuous variables are mean ±SD (median).

3 <sup>\*</sup>Values are presented as number (%).

4 <sup>†</sup>Values are presented as median (Q1~Q3)

5 <sup>#</sup>p values was calculated by chi-square statistical test for nominal variables, t-test/Wilcoxon  
6 rank sum test for continuous variables to assess whether there were significant differences  
7 between sex.



**Table 2.** Estimated Coefficient ( $\beta$ ) with 95%CI of natural log-transformed testosterone (nmol/L) per each 1 (ng/mL) increase in PFAS levels in multivariate linear regression models

	Total coefficient (95% CI) <sup>*</sup>	Boys coefficient (95% CI) <sup>†</sup>	Girls coefficient (95% CI) <sup>†</sup>	<i>P</i> -value for Interaction <sup>#</sup>
PFOS	-0.0022 (-0.0076 to 0.0032)	<b><u>-0.0029</u></b> <b><u>(-0.0055 to -0.0003)</u></b>	0.0005 (-0.0018 to 0.028)	0.060
PFOA	0.0642 (-0.0903 to 0.2186)	-0.0549 (-0.1186 to 0.0088)	-0.0697 (-0.1627 to 0.0233)	0.421
PFBS	-0.0391 (-0.6840 to 0.7622)	-0.0387 (-0.3261 to 0.2487)	0.1326 (-0.3576 to 0.6229)	0.457
PFDA	-0.1938 (-0.4842 to 0.0965)	<b><u>-0.2565</u></b> <b><u>(-0.4135 to -0.0994)</u></b>	-0.0626 (-0.1730 to 0.0477)	0.103
PFDoA	-0.0016 (-0.0257 to 0.0225)	0.0056 (-0.0056 to 0.0168)	<b><u>-0.0119</u></b> <b><u>(-0.0227 to -0.0010)</u></b>	0.119
PFHxA	-0.1817 (-0.7727 to 0.4092)	<b><u>-0.3095</u></b> <b><u>(-0.5942 to -0.0248)</u></b>	-0.1896 (-0.4387 to 0.0595)	0.476
PFHxS	-0.0040 (-0.0732 to 0.0652)	0.0173 (-0.0211 to 0.0588)	-0.0182 (-0.0451 to 0.087)	0.699
PFNA	-0.3413 (-0.8036 to 0.1210)	<b><u>-0.4233</u></b> <b><u>(-0.6998 to -0.1467)</u></b>	-0.1018 (-0.2684 to 0.0648)	0.042
PFTA	0.0006 (-0.0012 to 0.0024)	0.0009 (-0.0001 to 0.0019)	0.0003 (-0.0004 to 0.0009)	0.650

<sup>\*</sup>Models are adjusted for age, sex, BMI, ETS exposure, parental education, regular exercise, and month of survey.

<sup>†</sup>Models are adjusted for age, BMI, ETS exposure, parental education, regular exercise, and month of survey.

<sup>#</sup>*P* from the interaction term between PFASs and sex in joint models.

Coefficient represents the change in testosterone outcome for each 1 ng/mL increase in PFASs concentration.

**Table 3.** Estimated Coefficient ( $\beta$ ) with 95%CI of natural log-transformed estradiol (pmol/L) per each 1 (ng/mL) increase in PFAS levels in multivariate linear regression models

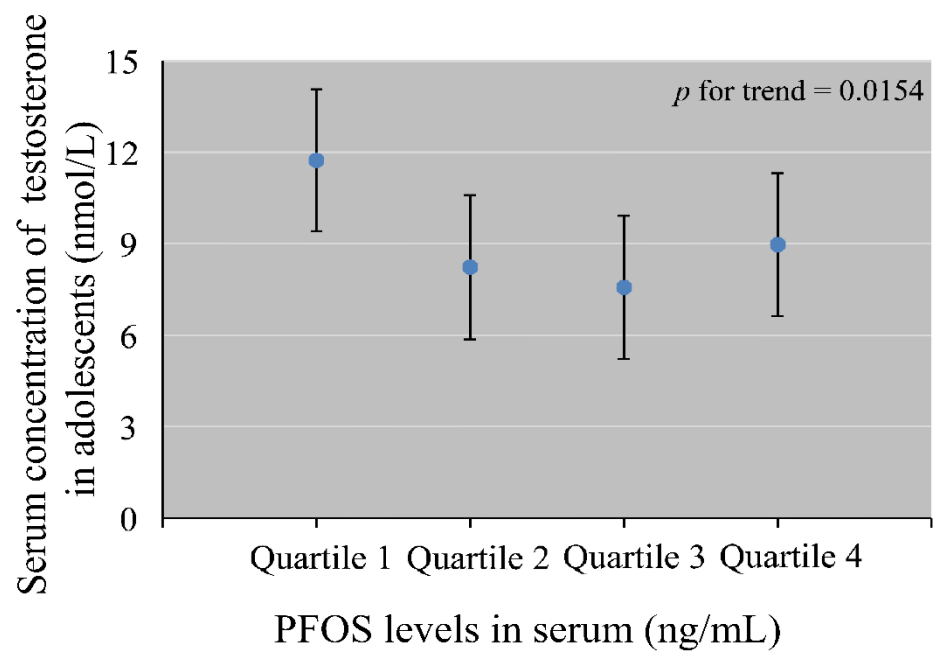
	Total coefficient (95% CI) <sup>*</sup>	Boys coefficient (95% CI) <sup>†</sup>	Girls coefficient (95% CI) <sup>†</sup>	<i>P</i> -value for Interaction <sup>#</sup>
PFOS	0.0018 (-0.0006 to 0.0042)	0.0024 (-0.0007 to 0.0055)	0.0005 (-0.0023 to 0.0033)	0.256
PFOA	0.0570 (-0.0112 to 0.1253)	<b>0.0921</b> <b>(0.0186 to 0.1656)</b>	0.1015 (-0.0103 to 0.2134)	0.475
PFBS	0.0447 (-0.2763 to 0.3656)	0.0149 (-0.3216 to 0.3513)	0.3129 (-0.2771 to 0.9028)	0.316
PFDA	0.0537 (-0.0756 to 0.1829)	0.0734 (-0.1189 to 0.2657)	0.0131 (-0.1208 to 0.1469)	0.615
PFDoA	0.0037 (-0.0070 to 0.0144)	-0.0007 (-0.0139 to 0.0124)	0.0106 (-0.0026 to 0.0238)	0.221
PFHxA	-0.0529 (-0.3154 to 0.2096)	0.0600 (-0.2803 to 0.4003)	-0.1492 (-0.4515 to 0.1531)	0.307
PFHxS	0.0249 (-0.0056 to 0.0555)	<b>0.0462</b> <b>(0.0020 to 0.0905)</b>	0.0171 (-0.0154 to 0.0496)	0.570
PFNA	<b>0.2060</b> <b>(0.0016 to 0.4105)</b>	0.3204 (-0.0115 to 0.6522)	0.1252 (-0.0758 to 0.3263)	0.478
PFTA	0.0003 (-0.0005 to 0.0011)	-0.0003 (-0.0014 to 0.0009)	0.0007 (-0.0001 to 0.0014)	0.215

<sup>\*</sup> Models are adjusted for age, sex, BMI, ETS exposure, parental education, regular exercise, and month of survey.

<sup>†</sup> Models are adjusted for age, BMI, ETS exposure, parental education, regular exercise, and month of survey.

<sup>#</sup> *P* from the interaction term between PFASs and sex in joint models.

Coefficient represents the change in estradiol outcome for each 1 ng/mL increase in PFASs concentration.



**Figure 1.** Serum concentration of testosterone among children according to quartiles of PFOS exposure. The data are expressed as estimated mean and 95% CI adjusted for All models are adjusted for age, gender, BMI, ETS exposure, parental education, regular exercise, and month of survey. p-Values for trend were calculated using categories representing the median value of the corresponding quartile.