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*Original Research*

# Association of Perfluoroheptanoic Acid With Total Testosterone: A Retrospective Study in Women of Childbearing Age and an Experimental Study in Female Mice Exposed to Perfluoroheptanoic Acid

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**Abstract**

**Background**: Multiple studies have demonstrated that exposure to perfluoroalkyl substances (PFAS) adversely affects endocrine function, reproductive health, metabolic processes, immune responses, and cardiovascular health; however, integration of population and animal studies remains limited. **Methods**: We collected clinical data and serum samples from 247 women of childbearing age who underwent pre-pregnancy fertility assessment at Hainan Women and Children's Medical Center from January 2022 to June 2024. Serum samples were collected to assess the levels of PFAS. Furthermore, twenty female C57BL/6J mice, aged 8 weeks, were randomly allocated into four groups: a control group receiving an equivalent volume of a 1% Tween and 2% dimethyl sulfoxide aqueous solution, a low-dose perfluoroheptanoic acid (PFHpA) group (0.5 mg/kg/day), a medium-dose PFHpA group (5 mg/kg/day), and a high-dose PFHpA group (50 mg/kg/day). Serum reproductive hormone levels and the expression of cytochrome P450 family 11 subfamily A polypeptide 1 (CYP11A1), cytochrome P450 family 17 subfamily A polypeptide 1 (CYP17A1), 3$\beta$-hydroxysteroid dehydrogenase (3$\beta$-HSD), and 17$\beta$-hydroxysteroid dehydrogenase (17$\beta$-HSD) in the mice were quantified using enzyme-linked immunosorbent assay (ELISA) and quantitative polymerase chain reaction (qPCR). The associations between individual PFAS compounds and reproductive hormones, as well as intergroup mean differences, were analyzed utilizing IBM SPSS Statistics version 27. **Results**: In the human study, the natural logarithm of PFHpA showed a positive association with total testosterone (TT) levels, with a $\beta$ of 0.51 (95% confidence interval [CI] for B: 0.18, 0.30). The high-dose PFHpA group exhibited elevated TT levels compared with all three other groups, with all $p$-values $< 0.0001$. Furthermore, in the animal study, CYP11A1 levels were increased in the high-dose PFHpA group compared with both the control and low-dose PFHpA groups, with all $p$-values $< 0.01$. Additionally, the high-dose PFHpA group demonstrated higher levels of CYP17A1, 3$\beta$-HSD, and 17$\beta$-HSD than all other groups, with all $p$-values $< 0.05$. **Conclusions**: This research concluded that PFHpA exposure affects TT levels. The increase in TT may result from upregulated expression of multiple enzymes within ovarian tissues, such as CYP11A1, CYP17A1, 3$\beta$-HSD, and 17$\beta$-HSD, following PFHpA exposure.

**Keywords:** perfluoroheptanoic acid; total testosterone; childbearing age; steroid pathway

## 1. Introduction

Perfluoroalkyl substances (PFAS) are synthetic organic compounds characterized by their robust carbon-fluorine bonds. They typically include at least one perfluorinated methyl group ($–CF_3$) or a perfluorinated methylene group ($–CF_2–$), with certain exceptions [1]. PFAS are generally classified as short-chain, which include seven or fewer carbon atoms, or long-chain, which include eight or more carbon atoms. They are often referred to as legacy compounds, which reflects the duration of chemical evaluation [2]. Due to their significant thermochemical stability and hydrophobic properties, PFAS are frequently present in consumer products and household items, particularly in furniture, carpets, and firefighting foam [3]. There are thousands of alkyl chain chemicals of variable length that contain a perfluoroalkyl segment (CnF2n+1–), and their environmental and toxicological properties have been extensively investigated [3,4]. Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS) have been widely detected in flora, fauna, human biological samples, and various soil and water specimens worldwide [5–8]. These substances have demonstrated multisystem toxicity, which arises primarily from prolonged exposure through various routes, including dietary intake, inhalation of airborne particles, and dermal contact [5–8]. Evidence indicates that PFAS exposure adversely impacts endocrine function, re-

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productive health, metabolic processes, immune system performance, and cardiovascular health [4,8–10]. Numerous institutions and regulatory organizations now enforce stricter controls on the production and use of PFAS [4]. Following the discontinuation of PFOA, PFOS, and perfluorohexanesulfonic acid (PFHxS), the global production of PFAS has transitioned toward alternative compounds [4]. This encompasses short-chain PFAS, including perfluorobutyric acid (PFBA), perfluorobutane sulfonic acid (PFBS), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), and perfluoroalkyl ether sulfonic acids (PFECAs). Some of these PFAS possess shorter alkyl chains or distinct chemical functional groups compared with traditional PFAS [11]. Although these alternative PFAS remain environmentally persistent, many appear less bioaccumulative and exhibit shorter elimination half-lives compared to legacy PFAS [12]. Among the PFECAs are hexafluoropropylene oxide dimer acid (HFPO-DA) and hexafluoropropylene oxide trimer acid (HFPO-TA), with HFPO-DA commonly referred to as GenX. Additionally, there are chlorinated perfluoroalkyl ether sulfonic acids (Cl-PFESAs), such as 6:2 Cl-PFESA and 8:2 Cl-PFESA.

PFAS persist in both the environment and biological organisms. Although some alternatives to PFAS have shorter half-lives in humans, they remain highly persistent in the environment, thereby posing ongoing exposure risks [13]. Between 2016 and 2021, concentrations of PFOS, PFBS, and HFPO-DA in rivers and lakes across China exhibited an upward trend [14]. Recent national water quality surveys indicate that levels of short-chain PFAS, particularly PFBA and PFBS, in water bodies are significantly higher than those of PFOA and PFOS [15]. Endocrine glands secrete hormones that regulate normal growth, fertility, and reproduction in humans [16]. Numerous studies have demonstrated that PFAS exposure can impair endocrine function and reproductive health [9,17]. Regarding the reproductive system, PFAS have the potential to interfere with endogenous hormone metabolism and the synthesis or secretion of steroid hormones, including estradiol ($E_2$), progesterone (P), total testosterone (TT), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) [2]. A moderate expression of cytochrome P450 family 11 subfamily A polypeptide 1 (CYP11A1), cytochrome P450 family 17 subfamily A polypeptide 1 (CYP17A1), 3$\beta$-hydroxysteroid dehydrogenase (3$\beta$-HSD), and 17$\beta$-hydroxysteroid dehydrogenase (17$\beta$-HSD) is essential for the regulation of female reproductive hormones and the synchronized release of gonadotropins [18–20]. However, studies integrating population and animal data remain limited. This research aims to validate the correlation between exposure to both traditional and emerging PFAS and reproductive hormones through population-based investigations, while establishing a mouse toxicity model to explore potential underlying mechanisms.

## 2. Materials and Methods

### *2.1 Study Subjects*

This research was a population-based study and was approved by the Medical Ethics Committee of Hainan Women and Children's Medical Center (Ethics Number: HNWCMC MEC No. 148 of 2025) in China. The study was conducted in strict accordance with the principles of the Declaration of Helsinki. Clinical data and serum samples were collected from 247 women of reproductive age who underwent preconception fertility evaluation at the Hainan Women and Children's Medical Center from January 2022 to June 2024. The inclusion criteria for all participants were as follows: (1) age between 20 and 35 years; (2) no history of chronic diseases, endocrine disorders, tumors, autoimmune diseases, or active infections; and (3) provision of informed consent to participate in the study. The exclusion criteria were as follows: (1) individuals with incomplete clinical information or missing samples; and (2) those who were pregnant, breastfeeding, or had a history of infertility. Data were collected on the participants' age, body mass index (BMI), age at menarche, number of pregnancies, menstrual cycle characteristics, income, and education level. Each woman underwent a comprehensive evaluation, which included measurement of FSH, LH, PRL, $E_2$, P, TT, and anti-müllerian hormone (AMH) during the mornings of days 2 to 4 of her menstrual cycle.

Twenty female C57BL/6J mice, each aged 8 weeks and weighing between 18 and 20 grams, purchased from GemPharmatech Co., Ltd., Nanjing, Jiangsu, China (License No. SCXK(SU)2023-0009), were utilized in this animal study. The sample size was determined based on the degrees of freedom derived from the variance analysis. All procedures and protocols involving mice were conducted in accordance with the guidelines of the Animal Care and Use Committee at Hainan Medical University and were approved by this committee (Ethics Number: HYLL-2024-222). All animals were housed in a controlled environment, with temperature, humidity, and light carefully regulated within a fully staffed, dedicated animal facility operating on a 12-hour light/dark cycle. Veterinarians were available on call at all times. Food and water were provided *ad libitum*. This research used Python 3.9 (Python Software Foundation, Beaverton, OR, USA) to calculate the required sample size for a one-way Analysis of Variance (ANOVA) power analysis. The anticipated effect size (Cohen's f) was established at 0.75, with a significance level of $\alpha = 0.05$ and a statistical power of 0.8, across four groups. Calculations demonstrated that a minimum of four mice per group was required to meet statistical criteria. To account for potential experimental attrition, the study ultimately included five mice per group, resulting in a total of twenty mice. Subsequently, a block randomization procedure utilizing Python 3.9 was implemented to generate a random allocation sequence, evenly distributing twenty mice into four groups of five subjects each. Following a one-week acclimatization

period, the mice were randomly allocated into four distinct groups: a control group administered an equal volume of a solution comprising 1% Tween and 2% dimethyl sulfoxide in water; a low-dose PFHpA group at 0.5 mg/kg/day; a medium-dose PFHpA group at 5 mg/kg/day; and a high-dose PFHpA group at 50 mg/kg/day. The doses were established following a comprehensive review of the existing literature [21–23]. Mice in the treatment cohort received daily oral gavage administrations of PFHpA for 28 consecutive days.

### *2.2 Measurements of Plasma Levels of PFAS Within the Population*

Blood samples were collected from women of reproductive age by venipuncture between days 2 and 4 of their menstrual cycle for preconception fertility evaluation. The samples were then centrifuged at 3000 rpm for 10 minutes at 4 °C and subsequently stored at –80 °C for PFAS analysis.

Methods for measuring PFAS were adapted from existing literature [24,25]. We utilized ultra-high-performance liquid chromatography-tandem quadrupole mass spectrometry (UHPLC-MS/MS) to analyze the concentrations of 22 different PFAS compounds in 0.1 mL of plasma. To ensure rigorous quality control, each batch of samples included internal quality control samples and procedural blank samples. Specifically, 2 ng of labeled standard and 1 mL of formic acid were added to the plasma samples. After vortexing, the samples were loaded onto the column and sequentially eluted with 1 mL of 0.1 M formic acid, 3 mL of a 50:50 mixture of 0.1 M formic acid and methanol, and 0.5 mL of a 1% ammonium hydroxide solution. PFAS were eluted using 1.8 mL of 1% ammonium hydroxide in acetonitrile. The eluate was then concentrated and diluted to 0.1 mL with a mixture of methanol and 10 mM ammonium acetate before transfer to a UPLC-MS/MS system for analysis. For the current study, only PFAS compounds with a detection rate exceeding 85% in the population were included in the analysis. This encompassed 1 short-chain PFAS (PFHpA), an emerging PFAS substitute (6:2 Cl-PFESA), and 8 legacy PFAS compounds (PFOA, PFOS, perfluorononanoic acid [PFNA], perfluorodecanoic acid [PFDA], PFHxS, perfluoroheptanesulfonic acid [PFHpS], perfluorododecanoic acid [PFDoA], and perfluoroundecanoic acid [PFUndA]). We defined the limit of detection (LOD) as the analyte concentration at which the signal-to-noise ratio (S/N) equals 3. Values below the LOD were substituted with the LOD divided by the square root of two ($\sqrt{2}$), which is an operation performed before the logarithmic transformation.

### *2.3 Measurements of Serum Reproductive Hormones, CYP11A1, CYP17A1, 3$\beta$-HSD, and 17$\beta$-HSD in Mice*

Two days after completing the oral gavage procedure, mice were anesthetized via intraperitoneal injection of a 0.3% sodium pentobarbital solution at a dose of 0.2 mL per 10 g of body weight. The procedure was initiated after the mice were placed under surgical anesthesia. Blood samples were collected via cardiac puncture and left at room temperature for 2 hours. The samples were then centrifuged at 1000 rpm for 20 minutes at 2 °C to 8 °C. The supernatants were then analyzed by enzyme-linked immunosorbent assay (ELISA) to quantify levels of FSH, LH, PRL, $E_2$, TT, and P. Subsequently, the mice were humanely euthanized via intraperitoneal injection of 3% sodium pentobarbital, administered at a dose of 0.05 mL per 10 grams of body weight.

Total RNA was extracted from mouse ovarian tissues on the same day using the Ultrapure RNA Kit (CW0581M, CWBIO, Taizhou, Jiangsu, China). RNA quality was verified by spectrophotometry, formaldehyde agarose gel electrophoresis, and spectrophotometric analysis. Expression analysis of transcripts related to the steroid pathway was performed using quantitative polymerase chain reaction (qPCR). In brief, cDNA was synthesized from 1 µg of total RNA using the HiScript II Q RT SuperMix (+gDNA wiper) (R223-01, Vazyme, Nanjing, Jiangsu, China) according to the manufacturer's instructions for qPCR. A PCR reaction mixture containing 1 µL of cDNA was amplified in a total volume of 20 µL using a PCR amplifier (TC-EA, Hangzhou BORI Technology Co., Ltd., Hangzhou, Zhejiang, China), with 0.8 µL of each forward and reverse primer pair (see Table 1). qPCR was performed using a fluorescent real-time PCR instrument (CFX Connect™ Real-Time, Bó Lè Life Medical Products (Shanghai) Co., Ltd., Shanghai, China). Relative fold gene expression changes ($2^{-\Delta\Delta CT}$) were calculated in comparison to vehicle controls and normalized to the expression of the reference gene. The transcripts analyzed included CYP11A1, CYP17A1, 3$\beta$-HSD, and 17$\beta$-HSD.

### *2.4 Statistical Analysis*

Mean values are expressed as the mean $\pm$ standard deviation (SD), while medians are presented as interquartile ranges (IQR). Categorical data are described using counts and percentages. PFAS concentrations are reported as medians and have been transformed with the natural logarithm to reduce skewness for subsequent analyses. Spearman correlation coefficients were calculated to assess the relationships among PFAS concentrations. Multiple linear regression models were used to examine the association between individual PFAS exposure and reproductive hormones. This study used a hybrid approach that combines prior knowledge with effect size change analysis to identify possible confounding factors. All regression analyses were adjusted for various general characteristics, including age, BMI, age at menarche, number of pregnancies, menstrual cycle irregularity, education, and income level. To manage the risk of false positives from multiple comparisons, we used the Benjamini-Hochberg (BH) method to control

**Table 1. Primer sequences for genes analyzed by qPCR.**

| Primers | Primer sequence (5′-3′) | Length (bp) | Annealing temperature (°C) |
|---|---|---|---|
| $\beta$-actin F | AGGGAAATCGTGCGTGAC | 192 | 51.6 |
| $\beta$-actin R | CATACCCAAGAAGGAAGGCT | | |
| CYP11A1 F | GAAGCGAGACTTCAGCCAGT | 132 | 58.0 |
| CYP11A1 R | AGGGTCATGGAGGTCGTGT | | |
| 3$\beta$-HSD F | AGCTCTGGACAAAGTATTCCGA | 234 | 58.0 |
| 3$\beta$-HSD R | GCCTCCAATAGGTTCTGGGT | | |
| CYP17A1 F | TCTGATACAAGCCAAGATGAATGC | 110 | 58.0 |
| CYP17A1 R | CCCCAAAGATGTCTCCCACC | | |
| 17$\beta$-HSD F | GTGGTTATGAGCAAGCCCTGAG | 112 | 58.0 |
| 17$\beta$-HSD R | CGGTTCGTGGAGAAGTAGCG | | |

qPCR, quantitative polymerase chain reaction; F, forward primer; R, reverse primer; CYP11A1, cytochrome P450 family 11 subfamily A polypeptide 1; 3$\beta$-HSD, 3$\beta$-hydroxysteroid dehydrogenase; CYP17A1, cytochrome P450 family 17 subfamily A polypeptide 1; 17$\beta$-HSD, 17$\beta$-hydroxysteroid dehydrogenase.

for the false discovery rate. Differences between group means were assessed using one-way ANOVA and post hoc tests or non-parametric methods. The significance level for all statistical analyses was established at 0.05 ($p < 0.05$). The analyses were performed utilizing IBM SPSS Statistics version 27 (IBM Corporation, Armonk, NY, USA), with graphical representations created using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

## 3. Results

### *3.1 Characteristics of the Study Population*

The demographic characteristics of the participants are summarized in Table 2. The mean age was 29.44 years, with a mean BMI of 21.41 kg/m$^2$ and a mean age at menarche of 13.15 years. Table 3 presents the median concentrations of reproductive hormones measured in the study, including FSH, LH, PRL, $E_2$, TT, P, and AMH. The median concentrations were 6.50 IU/L for FSH, 5.61 IU/L for LH, 19.90 ng/mL for PRL, 42.70 pg/mL for $E_2$, 0.28 ng/mL for TT, 0.22 ng/mL for P, and 3.80 ng/mL for AMH. The median levels of these reproductive hormones were all within the normal range for this age group. As shown in Table 4, the majority of women exhibited detectable levels of all 10 PFAS compounds assessed, with PFOA exhibiting the highest median concentration at 2.295 ng/mL.

**Table 2. Characteristics of the study population (n = 247).**

| Variable | Mean $\pm$ SD, or n (%) |
|---|---|
| Age (years) | 29.44 $\pm$ 2.67 |
| BMI (kg/m$^2$) | 21.41 $\pm$ 2.90 |
| Age at menarche (years) | 13.15 $\pm$ 0.92 |
| Number of pregnancies (%) | |
| Nulliparous | 120 (48.58%) |
| 1–2 | 102 (41.30%) |
| $\geq$3 | 25 (10.12%) |
| Menstrual cycle irregularity (%) | 42 (17.00%) |
| Education level | |
| Primary school | 46 (18.62%) |
| Middle school | 77 (31.17%) |
| Above middle school | 124 (50.20%) |
| Income level | |
| <7239 USD | 131 (53.04%) |
| $\geq$7239 USD | 116 (46.96%) |

SD, standard deviation; BMI, body mass index; USD, United States dollar.

### *3.2 Results of Multiple Linear Regression Analyses*

Table 5 presents the results of a linear regression analysis examining the associations between individual PFAS compounds and reproductive hormone levels. After adjusting for potential confounders, the natural logarithm of PFHpA was positively correlated with both $E_2$ and TT levels. Specifically, the $\beta$ coefficient for $E_2$ was 0.32, with a 95% confidence interval (CI) for B of 12.10 to 29.60 (B = 20.83). For TT, the $\beta$ was 0.51, with a 95% CI for B of 0.18 to 0.30 (B = 0.24). The natural logarithm of PFDA was also positively correlated with TT levels, exhibiting a $\beta$ of 0.32 and a 95% CI for B ranging from 0.01 to 0.40 (B = 0.20). No other PFAS showed significant associations with reproductive hormones.

### *3.3 Comparison of Serum Reproductive Hormone Levels Across Four Mice Groups*

Fig. 1 illustrates the serum reproductive hormone levels across four mice groups: the control group, the low-dose PFHpA group (0.5 mg/kg/day), the medium-dose PFHpA group (5 mg/kg/day), and the high-dose PFHpA group (50 mg/kg/day). The high-dose PFHpA group exhibited higher TT levels than all other three groups, with all comparisons yielding $p < 0.0001$. Additionally, the control group had lower PRL levels than the three other groups, with all $p < 0.01$. No significant differences were observed for the other reproductive hormones among the four groups.

**Table 3. Distribution of reproductive hormones (n = 247).**

| Reproductive hormones | LOD | Detection rate % | $P_{25}$ | $P_{50}$ | $P_{75}$ | Min | Max |
|---|---|---|---|---|---|---|---|
| FSH (IU/L) ($P_{25}$, $P_{75}$) | 0.10 | 100.00 | 5.64 | 6.50 | 7.35 | 3.78 | 21.52 |
| LH (IU/L) ($P_{25}$, $P_{75}$) | 0.10 | 100.00 | 4.15 | 5.61 | 7.32 | 1.61 | 19.77 |
| PRL (ng/mL) ($P_{25}$, $P_{75}$) | 1.00 | 100.00 | 14.90 | 19.90 | 27.80 | 5.70 | 76.20 |
| $E_2$ (pg/mL) ($P_{25}$, $P_{75}$) | 5.00 | 99.60 | 30.70 | 42.70 | 55.80 | 3.54 | 111.70 |
| TT (ng/mL) ($P_{25}$, $P_{75}$) | 0.03 | 100.00 | 0.19 | 0.28 | 0.37 | 0.03 | 0.70 |
| P (ng/mL) ($P_{25}$, $P_{75}$) | 0.05 | 96.40 | 0.14 | 0.22 | 0.32 | 0.04 | 2.02 |
| AMH (ng/mL) ($P_{25}$, $P_{75}$) | 0.01 | 100.00 | 2.19 | 3.80 | 5.46 | 0.13 | 12.23 |

LOD, limit of detection; FSH, follicle-stimulating hormone; LH, luteinizing hormone; PRL, prolactin; $E_2$, estradiol; TT, total testosterone; P, progesterone; AMH, anti-müllerian hormone.

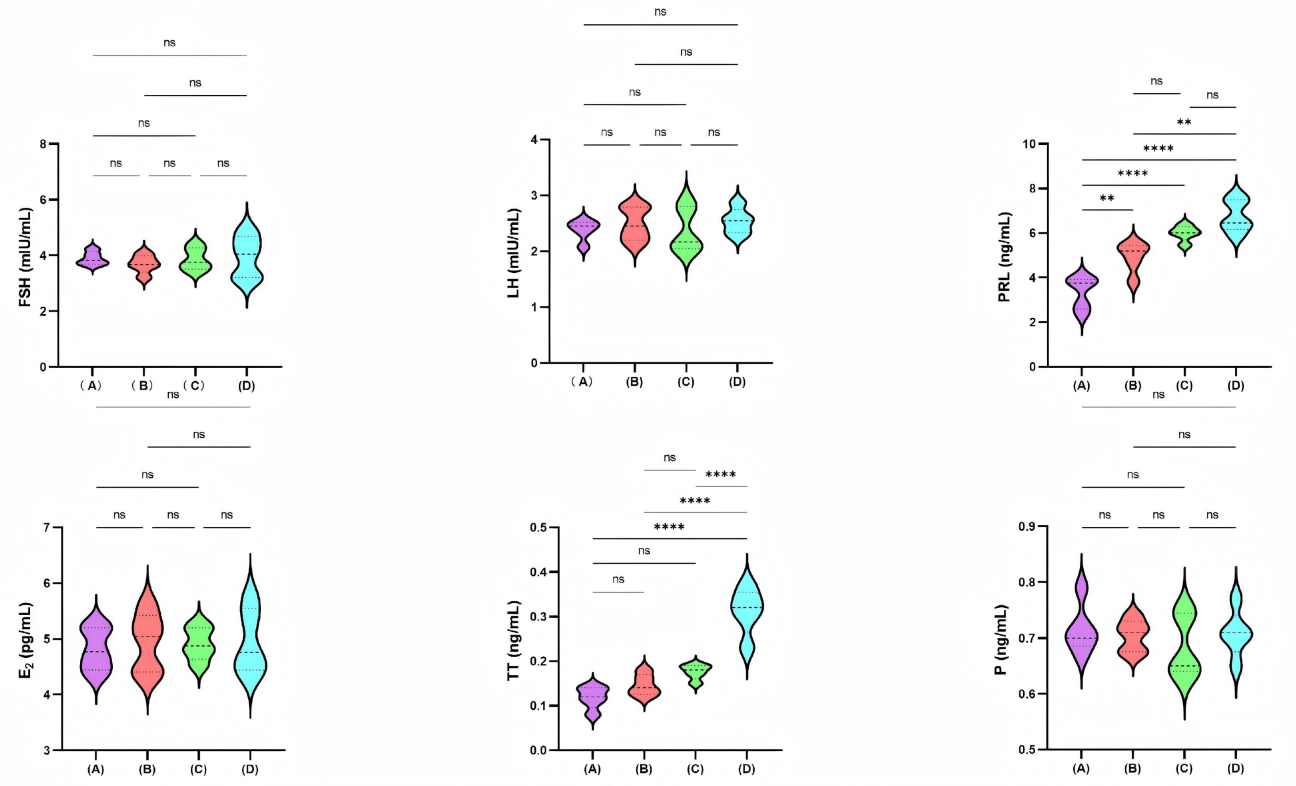


**Fig. 1. Comparison of serum reproductive hormone levels among the four mice groups.** A, Control group; B, Low-dose PFHpA group (0.5 mg/kg/day); C, Medium-dose PFHpA group (5 mg/kg/day); D, High-dose PFHpA group (50 mg/kg/day). ns: $p > 0.05$; **: $p < 0.01$; ****: $p < 0.0001$.

### *3.4 Comparison of CYP11A1, CYP17A1, 3β-HSD, and 17β-HSD Levels Across Four Mice Groups*

Fig. 2 shows the relative mRNA expression levels of CYP11A1, CYP17A1, 3$\beta$-HSD, and 17$\beta$-HSD across the four mice groups. Higher relative mRNA expression of CYP11A1 levels were observed in the high-dose PFHpA group compared to the control and low-dose PFHpA groups, with all comparisons yielding $p < 0.01$. Higher CYP11A1 levels were also observed in the medium-dose PFHpA than in the control groups. Furthermore, the high-dose PFHpA group exhibited higher CYP17A1, 3$\beta$-HSD, and 17$\beta$-HSD levels than all other groups, with all comparisons resulting in $p < 0.05$. Significant differences in 3$\beta$-HSD and 17$\beta$-HSD were also observed in the medium-dose PFHpA group compared to the other three groups, with $p$-values $< 0.05$ for all comparisons.

## 4. Discussion

Our findings examined the correlations between PFAS and reproductive hormones in women of reproductive age. The median concentrations of most PFAS detected in the serum of these women were lower than those reported in women of the same age group in other countries and regions of China [26,27]. We observed a positive association between exposure to PFHpA and TT levels, with a $\beta$ of 0.51 (95% CI for B: 0.18, 0.30). Similarly, our prior research

**Table 4. Distribution of plasma PFAS (n = 247).**

| PFAS (ng/mL) | LOD (ng/mL) | Detection rate % | $P_{25}$ (ng/mL) | $P_{50}$ (ng/mL) | $P_{75}$ (ng/mL) | Min (ng/mL) | Max (ng/mL) |
|---|---|---|---|---|---|---|---|
| 6:2 Cl-PFESA | 0.001 | 100.000 | 0.197 | 0.303 | 0.451 | 0.002 | 1.872 |
| PFOA | 0.005 | 100.000 | 1.555 | 2.295 | 3.237 | 0.140 | 13.384 |
| PFOS | 0.004 | 99.190 | 1.377 | 2.141 | 3.438 | <LOD | 9.156 |
| PFNA | 0.006 | 99.595 | 0.383 | 0.581 | 0.792 | <LOD | 2.598 |
| PFDA | 0.006 | 99.190 | 0.195 | 0.298 | 0.436 | <LOD | 1.566 |
| PFHxS | 0.004 | 99.595 | 0.182 | 0.318 | 0.466 | <LOD | 3.191 |
| PFHpS | 0.001 | 100.000 | 0.033 | 0.061 | 0.107 | 0.001 | 0.398 |
| PFDoA | 0.016 | 99.595 | 0.019 | 0.032 | 0.054 | <LOD | 0.298 |
| PFUndA | 0.012 | 99.595 | 0.249 | 0.411 | 0.672 | <LOD | 1.725 |
| PFHpA | 0.003 | 90.283 | 0.023 | 0.048 | 0.219 | <LOD | 1.775 |

PFAS, perfluoroalkyl substances; Cl-PFESA, chlorinated perfluoroalkyl ether sulfonic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; PFNA, perfluorononanoic acid; PFDA, perfluorodecanoic acid; PFHxS, perfluorohexanesulfonic acid; PFHpS, perfluoroheptanesulfonic acid; PFDoA, perfluorododecanoic acid; PFUndA, perfluoroundecanoic acid; PFHpA, perfluoroheptanoic acid.

**Table 5. Associations between individual PFAS and reproductive hormone levels analyzed by multivariable linear regression (n = 247)*.**

| | FSH $\beta$ (95% CI) | LH $\beta$ (95% CI) | PRL $\beta$ (95% CI) | $E_2$ $\beta$ (95% CI) | TT $\beta$ (95% CI) | P $\beta$ (95% CI) | AMH $\beta$ (95% CI) |
|---|---|---|---|---|---|---|---|
| 6:2 Cl-PFESA | 0.01 (–1.26, 1.40) | –0.02 (–2.30, 1.93) | 0.09 (–5.15, 13.50) | –0.10 (–20.30, 6.31) | –0.07 (–0.13, 0.05) | 0.06 (–0.12, 0.24) | –0.02 (–2.05, 1.59) |
| PFOA | –0.00 (–0.17, 0.17) | –0.02 (–0.30, 0.25) | –0.13 (–2.09, 0.33) | –0.09 (–2.67, 0.78) | –0.11 (–0.02, 0.00) | –0.03 (–0.03, 0.02) | –0.01 (–0.25, 0.22) |
| PFOS | 0.16 (–0.18, 0.50) | 0.01 (–0.53, 0.55) | –0.18 (–3.77, 1.03) | –0.04 (–3.86, 2.97) | –0.23 (–0.04, 0.01) | –0.32 (–0.09, 0.00) | –0.15 (–0.68, 0.25) |
| PFNA | 0.01 (–1.46, 1.53) | 0.12 (–1.51, 3.26) | 0.04 (–9.27, 11.80) | 0.13 (–8.62, 21.30) | 0.20 (–0.03, 0.17) | 0.11 (–0.13, 0.27) | 0.21 (–0.69, 3.40) |
| PFDA | –0.22 (–4.72, 1.06) | 0.17 (–2.33, 6.91) | 0.12 (–13.60, 27.20) | 0.02 (–27.70, 30.30) | **0.32 (0.01, 0.40)** | 0.17 (–0.20, 0.58) | 0.004 (–3.92, 4.01) |
| PFHxS | –0.04 (–1.12, 1.08) | –0.09 (–2.52, 0.99) | 0.07 (–5.07, 10.40) | 0.11 (–5.03, 17.00) | 0.05 (–0.05, 0.09) | –0.03 (–0.17, 0.13) | –0.09 (–2.18, 0.84) |
| PFHpS | 0.03 (–6.67, 8.16) | –0.20 (–21.51, 2.16) | 0.00 (–52.20, 52.30) | –0.06 (–92.50, 56.20) | –0.08 (–0.67, 0.32) | 0.05 (–0.81, 1.19) | –0.12 (–14.8, 5.48) |
| PFDoA | 0.03 (–11.17, 14.71) | 0.09 (–12.52, 28.78) | 0.03 (–80.04, 102.28) | –0.01 (–135.65, 123.70) | –0.01 (–0.90, 0.84) | –0.05 (–2.11, 1.39) | 0.17 (–4.54, 30.93) |
| PFUndA | 0.09 (–1.42, 2.47) | –0.11 (–4.17, 2.04) | –0.19 (–21.60, 5.85) | 0.07 (–15.20, 23.80) | –0.12 (–0.18, 0.08) | –0.04 (–0.30, 0.23) | –0.02 (–2.86, 2.48) |
| PFHpA | –0.11 (–1.56, 0.19) | 0.11 (–0.30, 2.50) | 0.01 (–5.62, 6.71) | **0.32 (12.10, 29.60)** | **0.51 (0.18, 0.30)** | 0.07 (–0.06, 0.18) | 0.12 (–0.21, 2.19) |

*Adjusted for age, BMI, age at menarche, number of pregnancies, menstrual cycle irregularity, education level, and income level. CI for non-standard coefficients. Bold characters indicate significance, $p < 0.05$. CI, confidence interval.

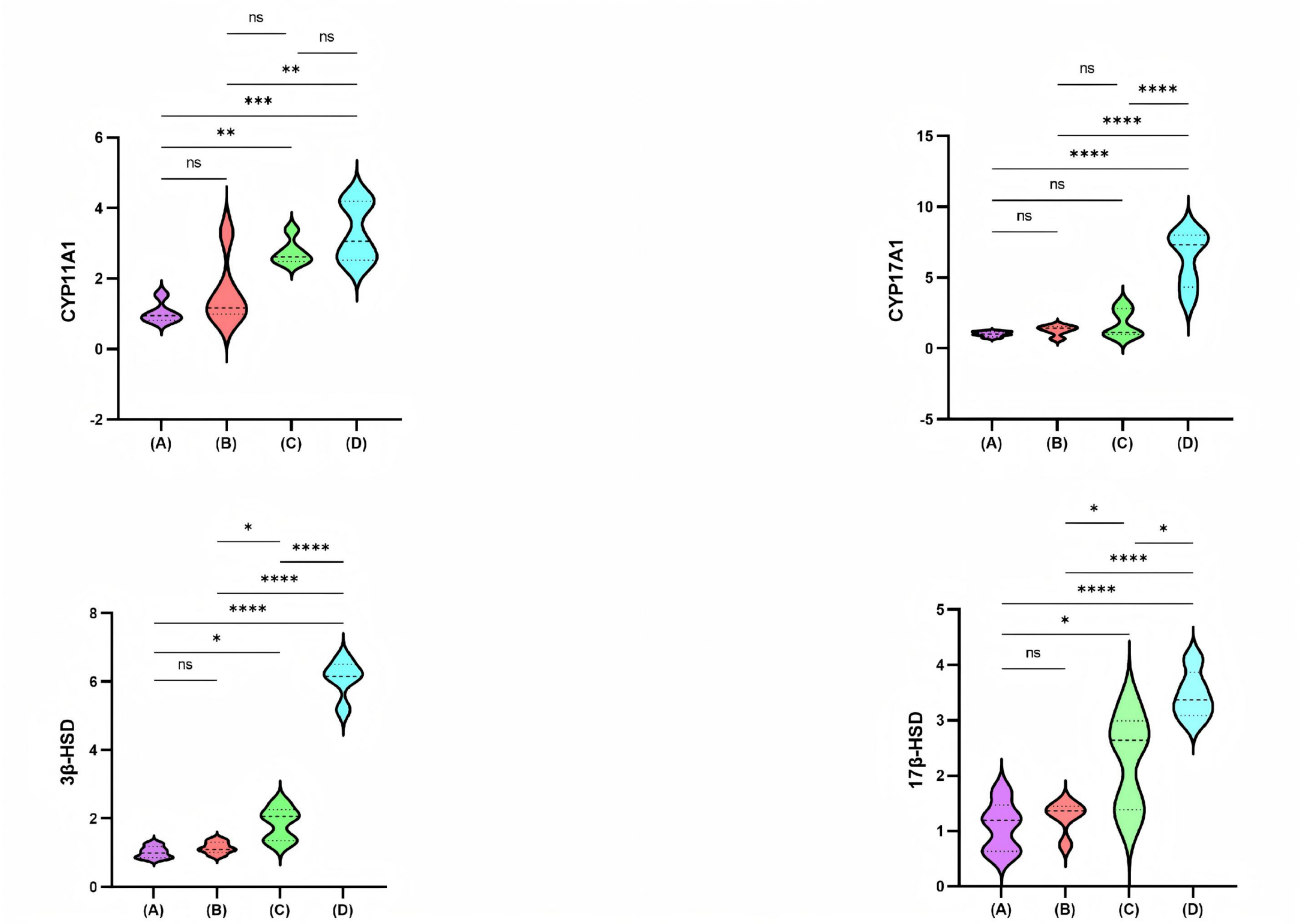


**Fig. 2. Comparison of CYP11A1, CYP17A1, 3$\beta$-HSD, and 17$\beta$-HSD levels across four mice groups.** A, Control group; B, Low-dose PFHpA group (0.5 mg/kg/day); C, Medium-dose PFHpA group (5 mg/kg/day); D, High-dose PFHpA group (50 mg/kg/day). ns: $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

also reported a positive correlation between PFHpA and TT levels [27]. To further investigate this relationship, we analyzed female mice exposed to PFHpA. Our results indicated significant increases in TT levels within the high-dose PFHpA group compared with the other three groups, with all $p$-values $< 0.0001$. PFHpA is widely used in non-stick coatings, fire extinguishing foams, electronic devices, textiles, food packaging, lubricants, and various chemical industries, primarily due to the global prohibition and gradual phase-out of PFOA and PFOS [28]. An analysis of hair samples collected from various populations in Spain indicated that PFHpA exhibited the highest detection rate among the six PFAS analyzed, reaching a maximum of 86% [29]. A prospective cohort study demonstrated that prenatal exposure to PFHpA significantly affected fetal gonadotropin and free androgen levels, resulting in reproductive toxicity in offspring [30]. A study evaluating PFHpA exposure in pregnant mice revealed that such exposure disrupts spermatogenesis and induces reproductive toxicity [31]. Exposure to PFHpA also increases the risk of gestational hypertension [32]. TT plays a vital role in the female reproductive system and functions as a biomarker for hyperandrogenism in women with hirsutism and amenorrhea [33,34]. TT is also a criterion utilized in the diagnosis of polycystic ovary syndrome (PCOS), the most common hormonal disorder among women of reproductive age [35]. In our population-based study, we also observed a positive association between exposure to PFHpA and $E_2$ levels, with a $\beta$ coefficient of 0.32 (95% CI for B: 12.10 to 29.60). However, this association was not observed in the model assessing PFHpA exposure in female mice. Consistent with our findings, evidence suggests that different types of PFAS exhibit variable relationships with $E_2$ levels in women of reproductive age [26,27,36].

PFHpA has emerged in recent years; however, research on its developmental and reproductive toxicity remains limited. A study involving rats demonstrated that exposure to PFHpA during puberty reduced the weights of the testes and epididymis. Furthermore, it induced hyperplasia of Leydig cells, potentially associated with decreased Bcl-2-associated X protein (BAX) levels, in conjunction with increased B-cell lymphoma 2 (BCL2) expression and phosphorylation of Extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and

AKT [22]. Key genes involved in steroidogenesis include CYP17A1, HSD17B5, and HSD17B6 [37]. A single-nucleotide polymorphism in the *CYP11A* gene leads to elevated androgen levels through the LH signaling pathway [38]. Post-translational modification of the *CYP17* gene also contributes to PCOS [39]. Reducing the expression of the *CYP11A1*, *CYP17A1*, and *DENND1A* genes decreases androgen production [39]. An *in vitro* study has shown that various PFAS notably increase the enzymatic activity of CYP enzymes [40]. In our study, CYP11A1 levels were significantly higher in the high-dose PFHpA group compared with the control and low-dose PFHpA groups, with all $p$-values $< 0.01$. Significant differences were also observed in the high-dose PFHpA group for CYP17A1, 3$\beta$-HSD, and 17$\beta$-HSD compared with the other three groups, with all pairwise comparisons yielding $p$-values $< 0.05$. Overall, the rise in TT may result from increased expression of several enzymes, including CYP11A1, CYP17A1, 3$\beta$-HSD, and 17$\beta$-HSD, in ovarian tissues of mice as PFHpA exposure levels increase.

*Limitations*

Our study has several limitations that warrant acknowledgment. First, reproductive hormone levels are known to fluctuate significantly over time, and these variations can markedly reduce the statistical power required to identify significant differences. Furthermore, the doses of PFHpA administered to the mice in this study were significantly higher than those typically encountered by the general population. This discrepancy may bias the evaluation of the causal relationship between PFHpA exposure and sex hormone levels, underscoring the need for further research.

## 5. Conclusions

This study revealed that PFHpA exposure affects TT levels in women of reproductive age. In female murine models, greater PFHpA exposure was associated with elevated TT levels. This increase in TT may result from upregulated expression of several ovarian enzymes, including CYP11A1, CYP17A1, 3$\beta$-HSD, and 17$\beta$-HSD, corresponding to increased PFHpA exposure. The findings provide both human and animal evidence linking PFHpA to reproductive hormones and establish the groundwork for further investigation into the underlying mechanisms.

## Availability of Data and Materials

The datasets used and analyzed in this study are available from the corresponding author upon reasonable request.

## Author Contributions

CYL was responsible for designing the study, conducting sample testing, analyzing data, drafting the original manuscript, and securing funding. ZYW contributed to sample testing, animal experimentation, and drafting the manuscript. HZW and XFF managed patient recruitment and specimen collection. SLZ assisted with data collection and analysis. HY played a key role in study design, data management, revising the manuscript, and obtaining funding. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

The population study received approval from the Medical Ethics Committee of Hainan Women and Children's Medical Center (Ethics Number: HNWCMC MEC No. 148 of 2025) in China and was conducted in accordance with the Declaration of Helsinki. Written informed consent for publication was obtained from all participants. All procedures and protocols for the animal experiment were conducted following the requirements of the Animal Care and Use Committee at Hainan Medical University, which approved this study (Ethics Number: HYLL-2024-222). The study was conducted and reported in accordance with the ARRIVE 2.0 guidelines.

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## Conflicts of Interest

The authors declare no conflicts of interest.

## Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/CEOG48278.

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