

# Exposure to Perfluoroalkyl Substances during Fetal Life and Pubertal Development in Boys and Girls from the Danish National Birth Cohort

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**BACKGROUND:** It remains unsettled whether prenatal exposure to perfluoroalkyl substances (PFASs) affects human reproductive health through potential endocrine disruption.

**OBJECTIVES:** We aimed to explore the associations between prenatal exposure to several PFASs and various aspects of pubertal development in boys and girls.

**METHODS:** We studied two samples ( $n = 722$  and  $445$ ) from the Puberty Cohort, nested within the Danish National Birth Cohort (DNBC), measuring PFAS in maternal plasma from early gestation. Data on pubertal development were collected biannually from the age of 11 y until full maturation, using web-based questionnaires. Outcomes were age at menarche, voice break, first ejaculation, and Tanner stages 2 to 5 for pubic hair, breast, genital development, and a combined puberty indicator. A regression model for censored data was used to estimate mean difference (months) in age at achieving the pubertal outcomes across tertiles of PFAS concentrations and with a doubling of PFAS concentrations (continuous). For perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), a meta-analysis was used to provide a weighted average of the point estimates from samples 1 and 2.

**RESULTS:** Overall, prenatal exposure to PFOS, perfluorohexane sulfonate (PFHxS), perfluoroheptane sulfonate (PFHpS), perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA) (girls) and PFHxS and PFHpS (boys) was associated with lower mean age at puberty marker onset. PFDA and PFNA exposure was associated with higher mean age at onset of puberty in boys. Nonmonotonic associations in girls (PFOS, PFHpS, PFDA) and boys (PFDA, PFNA) were observed, showing larger mean age differences for the combined puberty indicator in the middle tertile [girls: PFOS:  $-3.73$  mo, 95% confidence interval (CI):  $-6.59$ ,  $-0.87$ ; PFHpS:  $-4.92$  mo, 95% CI:  $-11.68$ ,  $1.85$ ; PFDA:  $-3.60$  mo, 95% CI:  $-9.03$ ,  $1.83$ ; and boys: PFNA:  $4.45$  mo, 95% CI:  $-1.30$ ,  $10.21$ ; PFDA:  $4.59$  mo, 95% CI:  $-0.93$ ,  $10.11$ ] than in the highest tertile with the lowest as reference.

**CONCLUSIONS:** Our population-based cohort study suggests sex-specific associations of altered pubertal development with prenatal exposure to PFASs. These findings are novel, and replication is needed. <https://doi.org/10.1289/EHP3567>

## Introduction

Perfluoroalkyl substances (PFASs) cover a group of synthetic chemicals that induce water, oil, and dirt resistance, and these are used in the manufacturing of a variety of everyday consumer products (Buck et al. 2011; Lindstrom et al. 2011). The substances are highly environmentally persistent and have long half-lives (Olsen et al. 2007), thus having the disadvantage of accumulating in humans (Calafat et al. 2007; Kannan et al. 2004) through various exposure routes, including drinking water, diet, dust, and air (Haug et al. 2011; Shoeib et al. 2011; Tittlemier et al. 2007).

PFASs cross the placental barrier (Apelberg et al. 2007; Inoue et al. 2004) and were recently detected in placental and fetal tissue from legally terminated pregnancies (Mamsen et al. 2017). This has raised concerns about the potential adverse effects of prenatal exposure to PFASs on later health, including reproductive health. These concerns are supported by an emerging number of *in vitro* and animal studies, proposing mechanisms by which PFASs may interfere with the endocrine system (Abbott 2009; Du et al. 2013; Kjeldsen and Bonefeld-Jørgensen 2013; Lau et al. 2007; Thibodeaux et al. 2003). Current epidemiologic literature mainly addresses PFAS exposure in adults and measures of fertility (Bach et al. 2016), prenatal exposure in relation to pregnancy outcomes (Fei et al. 2007; Olsen et al. 2009), and markers of short-term reproductive health, such as anogenital distance (Lind et al. 2017). In contrast, evidence on the potential impact of prenatal PFAS exposure on long-term reproductive health is still lacking, and previous studies have mainly included only perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) (Rappazzo et al. 2017).

The timing of puberty may be one of the biological processes vulnerable to endocrine disruption during fetal life due to the key role of the complex neuroendocrine regulation system in sexual maturation (Ebling 2005; Grumbach 2002). However, epidemiologic data on the potential adverse effects of prenatal exposure to PFASs on pubertal development remain sparse and inconsistent (Christensen et al. 2011; Kristensen et al. 2013; Lopez-Espinosa et al. 2011, 2016; Maisonet et al. 2015; Tsai et al. 2015; Vested et al. 2013), especially in boys, where only one study has been published (Vested et al. 2013). Existing studies have rather small sample sizes, and they include different markers of puberty timing, making it difficult to directly compare findings.

†This author drafted the article.

‡These authors conceptualized and designed the study.

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\*These authors contributed substantially to the interpretation of data, reviewed the article critically for important intellectual content, approved the final version to be published, and agree to be accountable for all aspects of the work.

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Thus, we explored the potential associations between prenatal exposure to several PFASs and a range of markers of pubertal development in boys and girls using two samples from a Danish nationwide puberty cohort with longitudinal data on puberty timing and plasma PFAS concentrations from first-trimester maternal samples.

## Methods

### Setting

The Danish National Birth Cohort (DNBC) is a nationwide cohort including 101,041 pregnancies in 91,661 women recruited between 1996 and 2002. The cohort holds data on health and lifestyle factors from a series of computer-assisted telephone interviews carried out twice during pregnancy and twice postpartum as well as blood samples from the pregnant women (Olsen et al. 2001).

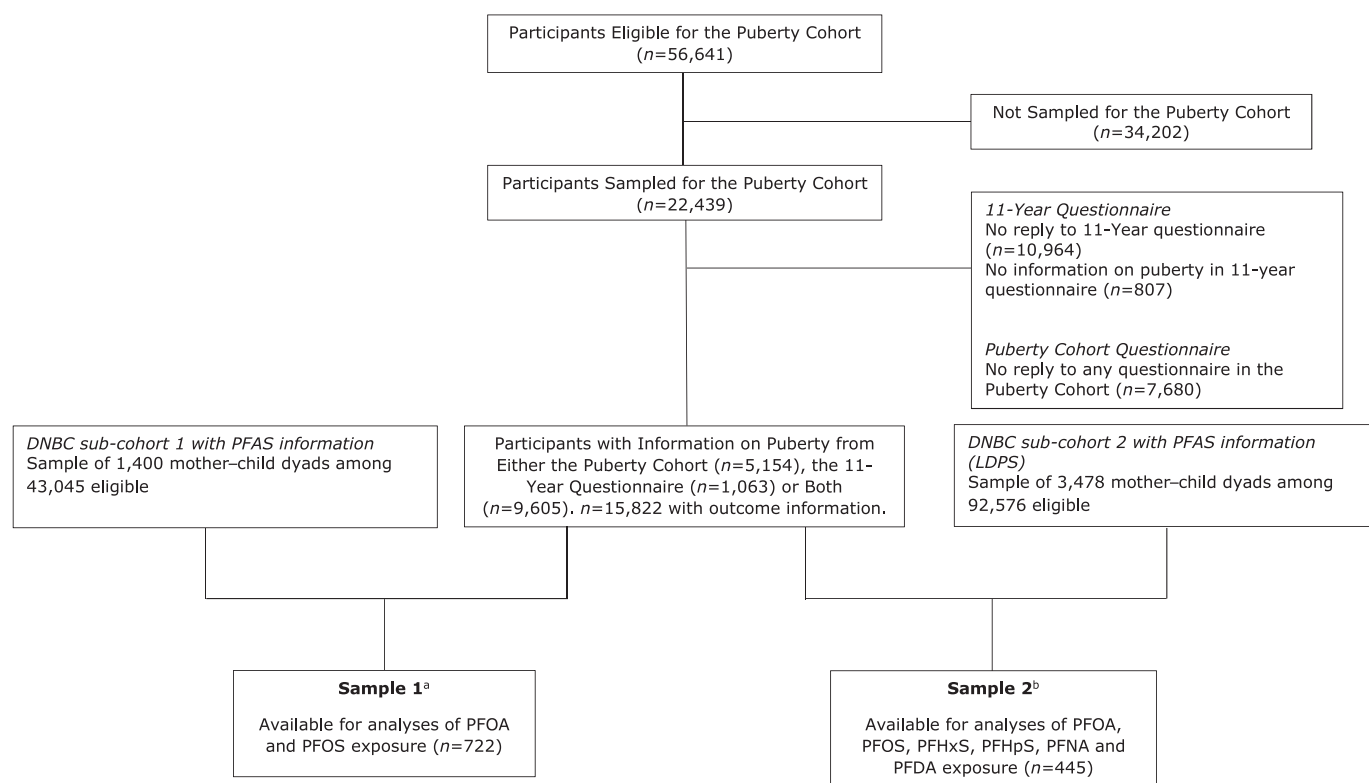
To follow up on the potential effect of intrauterine exposures on pubertal development, a subcohort, the Puberty Cohort, was established within the DNBC in 2012. Altogether, 56,641 live-born singletons born between 2000 and 2003 were eligible for participation, as their mothers responded to the first telephone interview and had not withdrawn from the DNBC before establishment of the Puberty Cohort in 2012. This Puberty Cohort was created by an oversampling strategy, aiming to increase statistical efficiency by ensuring sufficient sample sizes for a number of exposures of prior interest. We first sampled participants among eligible children according to subgroups of 12 different prenatal exposures (27 sampling frames). We also selected a random sample ( $n=8,000$ , one sampling frame) of the eligible children,

resulting in a total of 22,439 children being invited for participation in the Puberty Cohort (Figure 1). Detailed descriptions of the sampling strategy can be found elsewhere (Brix et al. 2018). In a 6-mo cycle, children were requested to provide detailed information on pubertal development in web-based questionnaires, performing self-assessment of their current pubertal stage. The data collection for the Puberty Cohort began at 11.5 y of age and ended when the child was fully sexual matured (defined as Tanner stage 5 for pubic hair development and genital/breast development) or turned 18 y of age, whichever came first. By March 2017, 14,759 of the 22,439 invited children had replied to the web-based questionnaires (66%).

In addition, all children in the DNBC had also been invited to complete a follow-up web-based questionnaire at 11 y of age. Among others, this questionnaire contained questions on pubertal development similar to the questions posed to participants in the Puberty Cohort. In the present study, we added the data on pubertal development from the 11-y questionnaire to the information available in the Puberty Cohort. Thus, information on pubertal development was available for 15,822 (7,697 boys and 8,125 girls) of the 22,439 children in the Puberty Cohort (71%) (Figure 1).

### Study Samples

Information on prenatal PFAS exposure was available from measurements in two other independently sampled DNBC subcohorts (Figure 1). In the study described by Fei et al. (2007), 1,400 mother–child dyads had PFAS measurements performed. Among those, 722 children also provided information on pubertal development in the Puberty Cohort (study sample 1). Further



**Figure 1.** Flowchart of study sample selection, Puberty Cohort, Denmark, 2012–2017 ( $n=722$  and  $445$ ). Note: DNBC, Danish National Birth Cohort; LDPS, Lifestyle During Pregnancy Study; PFDA, perfluorodecanoic acid; PFHpS, perfluoroheptane sulfonate; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid. <sup>a</sup>No perfluoroalkyl substance (PFAS) information available ( $n=14,422$ ), not in Puberty Cohort sample ( $n=412$ ), no reply to questionnaires in the Puberty Cohort ( $n=243$ ), and duplicate measurements with sample 2 ( $n=23$ ). <sup>b</sup>No PFAS information available ( $n=12,344$ ), not enrolled in LDPS or no PFAS measurements completed ( $n=1,884$ ), not in Puberty Cohort sample ( $n=1,072$ ), and no reply to questionnaires in the Puberty Cohort ( $n=77$ ).

information on pubertal development in 445 children with prenatal PFAS measurements from the Lifestyle During Pregnancy Study (LDPS) was available in the Puberty Cohort (study sample 2). The design of the LDPS has previously been described (Kesmodel et al. 2010). Briefly, the LDPS was originally created to examine the potential adverse effects of fetal exposure to maternal alcohol consumption and binge drinking patterns.

### Exposure Assessment

The majority of the women in the DNBC were in the first trimester when the general practitioners drew blood samples (median, gestational week 9; range, gestational week 5 to 25; interquartile range, gestational week 8 to 11) (Andersen et al. 2018) using ethylenediaminetetraacetic acid-coated tubes. The blood samples were sent by mail to Statens Serum Institut (Copenhagen, Denmark) ambient temperature, arriving within a median of 28 h later for storage in liquid nitrogen or freezers at  $-80^{\circ}\text{C}$ . Bach et al. (2015) investigated the impact of delays in processing and transportation on PFAS measurements. They reported that measurements in samples subjected to delays and transportation during the winter were generally lower compared to immediately processed samples. However, as any delayed handling and prolonged transportation time are considered unrelated to pubertal development, any measurement error would be nondifferential. The technique of solid phase extraction followed by liquid chromatography–tandem mass spectrometry was conducted to assess concentrations of PFASs in both samples 1 and 2.

The 3M Toxicology Laboratory (Mapplewood, Minnesota, United States) undertook the analyses of sample 1 (2007), measuring PFOA and PFOS, whereas the Department of Environmental Science at Aarhus University (Aarhus, Denmark) performed measurements of concentrations of 16 PFASs in sample 2 (2016), as analyses for more PFASs had become available at that time. Both laboratories were blinded to any information on mother and child. Further details on laboratory procedures and techniques have been described previously (Fei et al. 2007, 2009; Liew et al. 2014).

A total of 23 children with prenatal PFOA and PFOS measurements were available in both samples 1 and 2 (Figure 1). Since laboratory analyses were performed most recently in sample 2, we used levels of PFOA and PFOS from sample 2 in case of duplicates. Although the absolute PFOS and PFOA values in sample 1 were found to be slightly higher than sample 2, the correlations between the 23 duplicate samples were 0.95 (PFOA) and 0.92 (PFOS). Further, another study recently compared PFAS concentrations in a different set of blood samples from the DNBC analyzed at the two laboratories and found similarly high correlations, 0.94 (PFOS) and 0.95 (PFOA) (Liew et al. 2014). We included PFASs in the analyses of which 75% of the concentrations were quantifiable. In samples 1 and 2, PFOA and PFOS concentrations were all above the detection limit. In sample 2, four other PFASs [perfluorohexane sulfonate (PFHxS), perfluoroheptane sulfonate (PFHpS), perfluorononanoic acid (PFNA), and perfluorodecanoic acid (PFDA)] fulfilled the inclusion criteria. If PFAS concentrations were below the detection limit, this value was replaced by the lower limit of quantification divided by two. Table 1 shows the number of boys and girls for whom we needed to apply this strategy.

### Outcome Assessment

The biannual self-assessment by the children of current pubertal stage covered various secondary sexual characteristics including weight (kg), height (cm), pubic hair growth, and genital or breast growth categorized by Tanner rating scales (1–5) (Marshall and Tanner 1969, 1970), occurrence of acne (no/yes), and axillary

**Table 1.** Detection limits and sample-specific distribution of plasma perfluoroalkyl substance (PFAS) concentrations in first-trimester maternal samples from participants in the puberty cohort ( $n = 722$  and 445), stratified by sex, Denmark (2017).

| Analyte      | Sample 1 ( $n = 722$ ) <sup>a</sup> |             |                              |                                       | Sample 2 ( $n = 445$ ) <sup>b</sup> |                                       |                              |                                       |
|--------------|-------------------------------------|-------------|------------------------------|---------------------------------------|-------------------------------------|---------------------------------------|------------------------------|---------------------------------------|
|              | Boys ( $n = 356$ )                  |             | Girls ( $n = 366$ )          |                                       | Boys ( $n = 235$ )                  |                                       | Girls ( $n = 210$ )          |                                       |
| Abbreviation | Chemical name                       | LOD (ng/mL) | <LOD [ $n$ (%)] <sup>b</sup> | Median (10th–90th percentile) (ng/mL) | <LOD [ $n$ (%)] <sup>b</sup>        | Median (10th–90th percentile) (ng/mL) | <LOD [ $n$ (%)] <sup>b</sup> | Median (10th–90th percentile) (ng/mL) |
| PFOA         | Perfluorooctanoic acid              | 0.07        | 0                            | 5.1 (2.8–8.3)                         | 0                                   | 4.3 (2.2–6.7)                         | 0                            | 4.1 (2.3–6.4)                         |
| PFOS         | Perfluorooctanesulfonic acid        | 0.09        | 0                            | 31.9 (19.2–51.2)                      | 0                                   | 27.2 (16.7–45.2)                      | 0                            | 27.9 (16.5–42.2)                      |
| PFHxS        | Perfluorohexane sulfonate           | 0.03        | NA                           | NM                                    | NA                                  | 1.1 (0.6–1.7)                         | 0                            | 1.0 (0.6–1.6)                         |
| PFHpS        | Perfluoroheptane sulfonate          | 0.04        | NA                           | NM                                    | 1 (0.4)                             | 0.4 (0.2–0.6)                         | 3 (1.4)                      | 0.4 (0.2–0.6)                         |
| PFNA         | Perfluorononanoic acid              | 0.09        | NA                           | NM                                    | 7 (3.0)                             | 0.5 (0.3–0.7)                         | 10 (4.8)                     | 0.5 (0.3–0.8)                         |
| PFDA         | Perfluorodecanoic acid              | 0.03        | NA                           | NM                                    | 4 (1.7)                             | 0.2 (0.1–0.3)                         | 3 (1.4)                      | 0.2 (0.1–0.3)                         |

Note: —, data not available; LOD, lower limit of detection; NA, not available; NM, not measured.

<sup>a</sup>Maternal blood samples from early pregnancy collected in the Danish National Birth Cohort (DNBC) from 2000 to 2002.

<sup>b</sup>Number of sample-specific observations with concentrations below LOD.

hair growth (no/yes). Further, boys were asked to report occurrence of voice break [no; yes, sometimes (considered as voice break); yes, definitively; do not know) and age at first nocturnal ejaculation by year and months. Similarly, girls provided information on first menstrual bleeding by year and months. In each questionnaire, participants were asked to mark their current stage of Tanner pubic hair growth and genital or breast development, and indicate whether they had experienced the other indicators of pubertal development. When participants attained Tanner stage 5 or one of the other pubertal indicators, those specific questions were removed from the subsequent questionnaire. Our web-based questionnaire was inspired by the questionnaire used in the British Avon Longitudinal Study of Parents and Children (ALSPAC) (Monteilh et al. 2011). This is available in Danish at [www.bsig.dk](http://www.bsig.dk). Tanner staging was supported by pictograms and explained by descriptive texts.

### Covariates

We identified potential confounding factors *a priori* using existing literature and directed acyclic graphs (Figure S1). As the analyses were limited by sample size, we had to prioritize between potential confounders, and included the following in all adjusted models: highest social class of parents, maternal age at menarche, maternal age at delivery, parity, prepregnancy body mass index (BMI), and smoking during first trimester. The categorization of the included covariates can be found in Table 2 except for prepregnancy BMI, which was included as a second-order polynomial. Statistics Denmark provided data on highest social class of parents by the International Standard Class of Occupation and Education codes (ISCO-88 and ISCED), whereas

information on the remaining covariates were available from either the telephone interviews within the DNBC framework or the Danish Medical Birth Register. In the DNBC, the mothers were asked to report age at menarche in year. If they were unable to specify in years, they were asked to report at which grade that they experienced menarche. Lastly, if they were still unable to specify, the mothers indicated whether they experienced age at menarche earlier, at the same time, or later than peers. Those with information in year or grade were subsequently recategorized as earlier, same time, or later than peers according to national values from the Danish Ministry of Education.

### Statistics

We performed statistical analyses in each sample separately. PFOA and PFOS concentrations were measured in both sample 1 and sample 2, and the sample-specific estimates (see sample-specific estimates in Tables S1 and S2) were combined in a meta-analysis [the “metan” command in Stata/MP (version 15; StataCorp)] (Palmer and Sterne 2016) to better account for the possible difference in sampling criteria and laboratory setting. PFAS concentrations in each sample were treated as categorical variables divided into tertiles, with the lowest tertile serving as the reference group (Table 1: detection limits and sample-specific distribution of serum PFAS concentrations; Table 3: sample-specific tertile cut points) and continuous, per doubling of the predictor variable (log2 transformation).

In each sample, the analyses were conducted by a censored regression model for normally distributed time-to-event-data (the “intreg” command in Stata/MP), using inverse probability weights calculated from the specific sampling fractions in the two

**Table 2.** Description of study samples ( $n = 722$  and  $445$ ), Puberty Cohort, Denmark, 2017.

| Covariates                                             | Sample 1 ( $n = 722$ ) | Missing (%) | Sample 2 ( $n = 445$ ) | Missing (%) |
|--------------------------------------------------------|------------------------|-------------|------------------------|-------------|
| Sex, $n$ (%)                                           | —                      | 0 (0)       | —                      | 0 (0)       |
| Male                                                   | 356 (49.3)             | —           | 235 (52.8%)            | —           |
| Female                                                 | 366 (50.7)             | —           | 210 (47.2%)            | —           |
| Parity, $n$ (%)                                        | —                      | 0 (0)       | —                      | 0 (0)       |
| First child                                            | 342 (47.4)             | —           | 215 (48.3)             | —           |
| Second or more child                                   | 380 (52.6)             | —           | 230 (51.7)             | —           |
| Maternal age at delivery in years, mean $\pm$ SD       | 30.8 $\pm$ 4.3         | 0 (0)       | 31.1 $\pm$ 4.5         | 0 (0)       |
| Maternal age at menarche, $n$ (%) <sup>a</sup>         | —                      | 7 (1.0)     | —                      | 3 (0.7)     |
| Earlier than peers                                     | 175 (24.5)             | —           | 123 (27.8)             | —           |
| Same time as peers                                     | 410 (57.3)             | —           | 242 (54.8)             | —           |
| Later than peers                                       | 130 (18.2)             | —           | 77 (17.4)              | —           |
| Highest social class of parents, $n$ (%) <sup>b</sup>  | —                      | 2 (0.3)     | —                      | 1 (0.2)     |
| High-grade professional                                | 171 (23.8)             | —           | 113 (25.5)             | —           |
| Low-grade professional                                 | 254 (35.3)             | —           | 154 (34.7)             | —           |
| Skilled worker                                         | 203 (28.2)             | —           | 119 (26.8)             | —           |
| Unskilled worker                                       | 92 (12.8)              | —           | 58 (13.1)              | —           |
| Daily number of cigarettes in first trimester, $n$ (%) | —                      | 5 (0.7)     | —                      | 2 (0.5)     |
| Nonsmoker                                              | 564 (78.7)             | —           | 288 (65.0)             | —           |
| 0–9 cigarettes/d                                       | 128 (17.9)             | —           | 117 (26.4)             | —           |
| > 10 cigarettes/day                                    | 25 (3.5)               | —           | 38 (8.6)               | —           |
| Alcohol units/wk in first trimester, $n$ (%)           | —                      | 0 (0)       | —                      | 0 (0)       |
| 0 units                                                | 385 (53.3)             | —           | 168 (37.8)             | —           |
| > 0–1 unit                                             | 241 (33.4)             | —           | 108 (24.3)             | —           |
| > 1–3 units                                            | 77 (10.7)              | —           | 48 (10.8)              | —           |
| > 3 units                                              | 19 (2.6)               | —           | 121 (27.2)             | —           |
| Prepregnancy BMI ( $\text{kg}/\text{m}^2$ ), $n$ (%)   | —                      | 21 (2.9)    | —                      | 5 (1.1)     |
| < 18.5                                                 | 26 (3.7)               | —           | 26 (5.9)               | —           |
| 18.5 to < 25                                           | 479 (68.3)             | —           | 286 (65.0)             | —           |
| 25 to < 30                                             | 155 (22.1)             | —           | 88 (20.0)              | —           |
| 30 +                                                   | 41 (5.8)               | —           | 40 (9.1)               | —           |
| 7-y BMI ( $\text{kg}/\text{m}^2$ ), mean $\pm$ SD      | 15.6 (1.7)             | 191 (26.5)  | 15.5 (1.8)             | 99 (22.2)   |

Note: —, data not available; BMI, body mass index; SD, standard deviation.

<sup>a</sup>Maternal age at menarche (AAM) reported in years. If unable to specify in years, then asked to report at which grade or, lastly, whether AAM was experienced earlier, same time, or later than peers. Observations with information in years or grade recategorized as earlier, same time, or later than peers according to national values from the Danish Ministry of Education.

<sup>b</sup>Based on the International Standard Class of Occupation and Education classification system (ISCO-88 and ISCED codes) from Statistics Denmark.



**Table 3.** Sample-specific tertile cut points of plasma perfluoroalkyl substance (PFAS) concentrations in first-trimester maternal samples from participants in the Puberty Cohort ( $n = 722$  and  $445$ ), Denmark (2017).

| Analyte | PFAS concentrations (ng/mL) |                      |           |                      |            |                      |                        |                      |           |                      |           |                      |
|---------|-----------------------------|----------------------|-----------|----------------------|------------|----------------------|------------------------|----------------------|-----------|----------------------|-----------|----------------------|
|         | Sample 1 ( $n = 722$ )      |                      |           |                      |            |                      | Sample 2 ( $n = 445$ ) |                      |           |                      |           |                      |
|         | Low                         | Boys/girls ( $n^a$ ) | Medium    | Boys/girls ( $n^a$ ) | High       | Boys/girls ( $n^a$ ) | Low                    | Boys/girls ( $n^a$ ) | Medium    | Boys/girls ( $n^a$ ) | High      | Boys/girls ( $n^a$ ) |
| PFOA    | 1.01–4.16                   | 112/129              | 4.17–5.87 | 119/123              | 5.89–41.5  | 125/114              | 1.03–3.36              | 78/68                | 3.4–4.88  | 79/73                | 4.9–13.8  | 78/69                |
| PFOS    | 6.4–28.0                    | 119/126              | 28.1–38.4 | 122/119              | 38.5–106.7 | 115/121              | 7.68–23.2              | 74/71                | 23.3–31.5 | 81/69                | 31.6–91.4 | 80/70                |
| PFHxS   | NM                          | NA                   | NM        | NA                   | NM         | NA                   | 0.11–0.87              | 77/80                | 0.88–1.26 | 87/62                | 1.27–5.73 | 71/68                |
| PFHpS   | NM                          | NA                   | NM        | NA                   | NM         | NA                   | 0.07–0.29              | 79/72                | 0.3–0.42  | 85/70                | 0.43–1.52 | 71/68                |
| PFNA    | NM                          | NA                   | NM        | NA                   | NM         | NA                   | 0.14–0.41              | 72/76                | 0.42–0.53 | 80/66                | 0.54–2.23 | 83/68                |
| PFDA    | NM                          | NA                   | NM        | NA                   | NM         | NA                   | 0.08–0.15              | 76/75                | 0.16–0.21 | 82/72                | 0.22–0.9  | 77/63                |

Note: —, data not available; NA, not available; NM, not measured; PFDA, perfluorodecanoic acid; PFHpS, perfluoroheptane sulfonate; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid.

<sup>a</sup>Number of boys and girl in sample-specific tertile.

subcohorts that provided PFAS measurements in this study. The inverse probability weights account for the original sampling strategies used to create these subcohorts. See detailed description elsewhere (Brix et al. 2018). In addition, robust standard errors were included in all the models to account for clustering of siblings [sample 1 ( $n = 2$ ) and sample 2 ( $n = 4$ )] and the use of inverse probability weights. We estimated the mean monthly differences with 95% confidence intervals (CI) in age at attaining the markers of pubertal development across levels of exposure. We applied the robust variance estimation (Huber 1967; White 1980) to estimate the associations between tertiles of PFAS concentration (one for each PFAS) and a combined puberty indicator in one model for each sex. These analyses assume homogeneity of effect for each studied PFAS and address the correlation structure in age at attaining the different milestones within individuals. Lastly, we performed a posthoc defined model check of the linear effect of PFAS by modeling the continuous PFAS concentrations as a second-order polynomial and test for quadratic departure from linearity. All analyses were performed separately for boys and girls. The adjusted results are presented for complete case, as the number of missing covariates was low (Table 2).

Due to the longitudinal, biannual collection of data in the Puberty Cohort, data were censored: left censored if the puberty indicator was attained before replying to the first questionnaire, interval censored if attained between two subsequent questionnaires, and right censored if not achieved at the time of returning the last questionnaire. The regression model handles the censoring of data by assuming that the distribution of age at achieving a given pubertal marker follows a normal distribution. This type of analytic strategy is recommended as the primary approach in studies with multiple longitudinally collected measurements of pubertal development (Euling et al. 2008).

We checked the assumption of normality of the interval-censored residuals by visually comparing a stepwise cumulative incidence function fitted by a nonparametric distribution estimator [the “icenreg” command in R (version 3.3.1; R Development Core Team)] with an incidence function based on the normal distribution. To check the assumption of constant variance of the residuals, these plots were subsequently divided across levels of covariates included in the models. These assumptions were fulfilled for all models (data not shown).

## Ethics

The Committee for Biomedical Research Ethics in Denmark approved the collection of research data in the DNBC [(KF) 01-471/94]. The steering committee of DNBC (2012-04 and 2015-47) approved the present study registered at the Danish Data

Protection Agency (2012-41-0379 and 2015-57-0002). The mothers gave written informed consents on behalf of themselves and their children prior to enrollment in the DNBC.

## Results

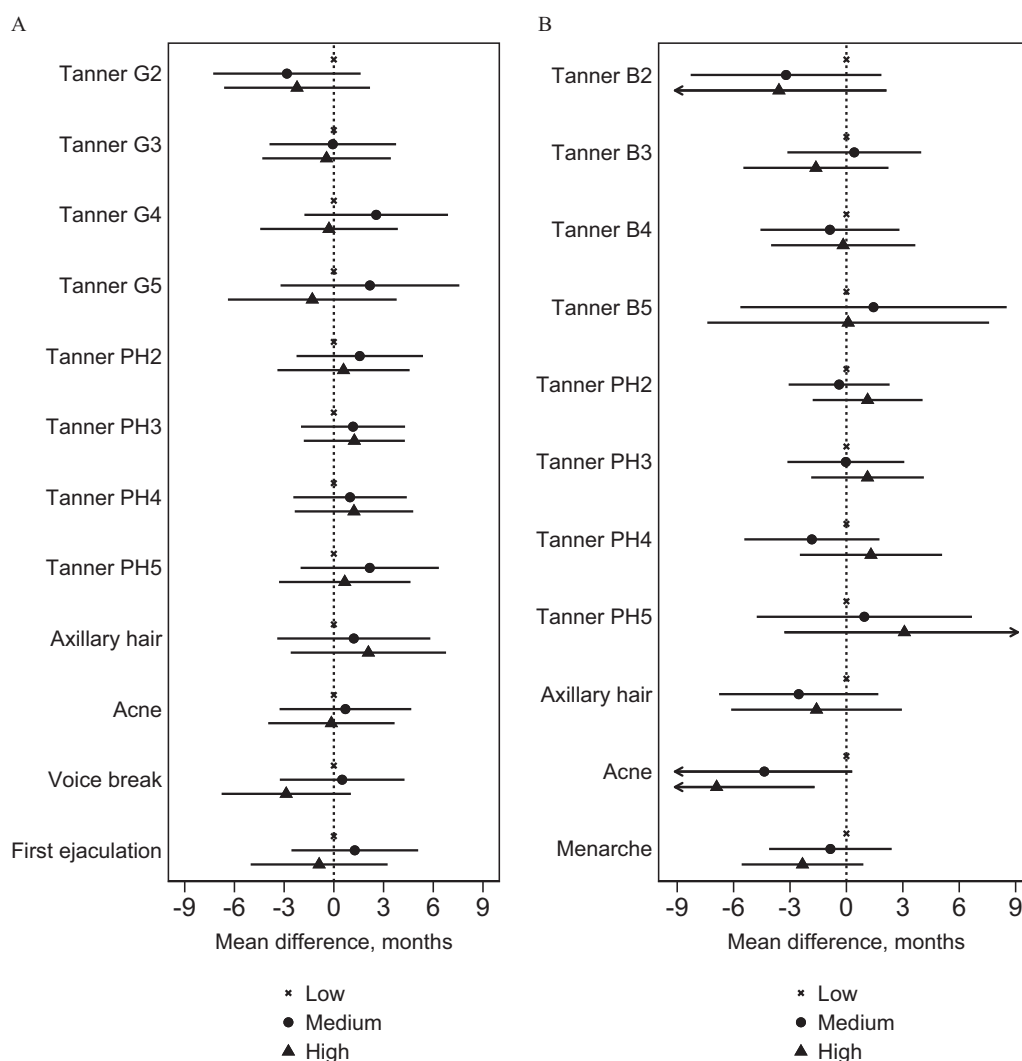
Per design, sample 2 included a larger proportion of mothers consuming alcohol and smoking during first trimester than sample 1 due to the sampling strategy used in LDPS (Table 2). Distributions of the remaining covariates were fairly comparable between the samples.

Table 1 lists the sample-specific and sex-stratified distributions of the six PFAS serum concentrations included in this study. Overall, the absolute PFOA and PFOS concentrations were lower on average in sample 1 compared to sample 2. However, the correlations of PFOS and PFOA were high in the 23 subjects that were part of both subcohorts. Within each sample, no sex-specific differences in the distribution of PFAS concentrations were observed.

### Perfluorooctanoic Acid and Perfluorooctanesulfonic Acid

Overall, no consistent pattern of associations between tertiles and continuous PFOA concentration and markers of male or female pubertal development was present (Figure 2A–B; Table 4; Table 5; and Table S3). The combined puberty indicator includes all puberty markers in one model stratified by sex and associations with tertiles of PFOA were close to the null in both boys and girls (boys: 0.71 mo, 95% CI: –1.88, 3.29, and –0.08 mo, 95% CI: –2.56, 2.40 in the middle and highest tertiles, respectively; and girls: –0.64 mo, 95% CI: –3.43, 2.16; and –0.39 mo, 95% CI: –3.25, 2.47 in the middle and highest tertiles, respectively).

For PFOS in girls, the middle vs. lowest tertile of exposure was associated with an earlier average age of onset for all individual outcomes except acne (CIs did not include null for Tanner breast stages 2–4, e.g., Tanner breast stage 2: –7.55 mo, 95% CI: –12.85, –2.24), while mean ages of onset for those in the highest vs. lowest PFOS tertile were also lower but closer to the null for all Tanner breast stages and acne onset, and mostly null or positive for the other outcomes (Figure 2D; Table S3). In girls, the mean ages of onset in association with a doubling of prenatal PFOS were lower (but CIs included the null) for Tanner breast stages (e.g., stage 2: –3.08, 95% CI: –7.15, 0.98), acne, and menarche, and null or positive for Tanner pubic hair stages and axillary hair growth (Table 4). A potential nonmonotonic exposure response between PFOS and puberty markers in girls was detected for 6 out of 11 outcomes ( $p$ -value < 0.05). For the combined female puberty indicator, the mean age of puberty timing was lower in the middle vs. the lowest PFOS tertile (–3.73 mo, 95% CI: –6.59, –0.87) and null for the highest vs. the lowest tertile (Table 5).



**Figure 2.** Estimated average difference (with 95% confidence intervals) in age (months) when pubertal milestones were attained according to tertiles of plasma PFOA and PFOS concentrations in first-trimester maternal samples. All estimates are adjusted for highest social class of parents, maternal age at menarche, maternal age at delivery, parity, prepregnancy body mass index, and maternal smoking during first trimester. (A) PFOA in boys. (B) PFOA in girls. (C) PFOS in boys. (D) PFOS in girls. See Table S3 for corresponding numeric data and Table 2 for tertile cutoff points. Note: PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; Tanner B2-5, Tanner breast stage 2-5; Tanner G2-5, Tanner genital stage 2-5; Tanner PH2-5, Tanner pubic hair stage 2-5.

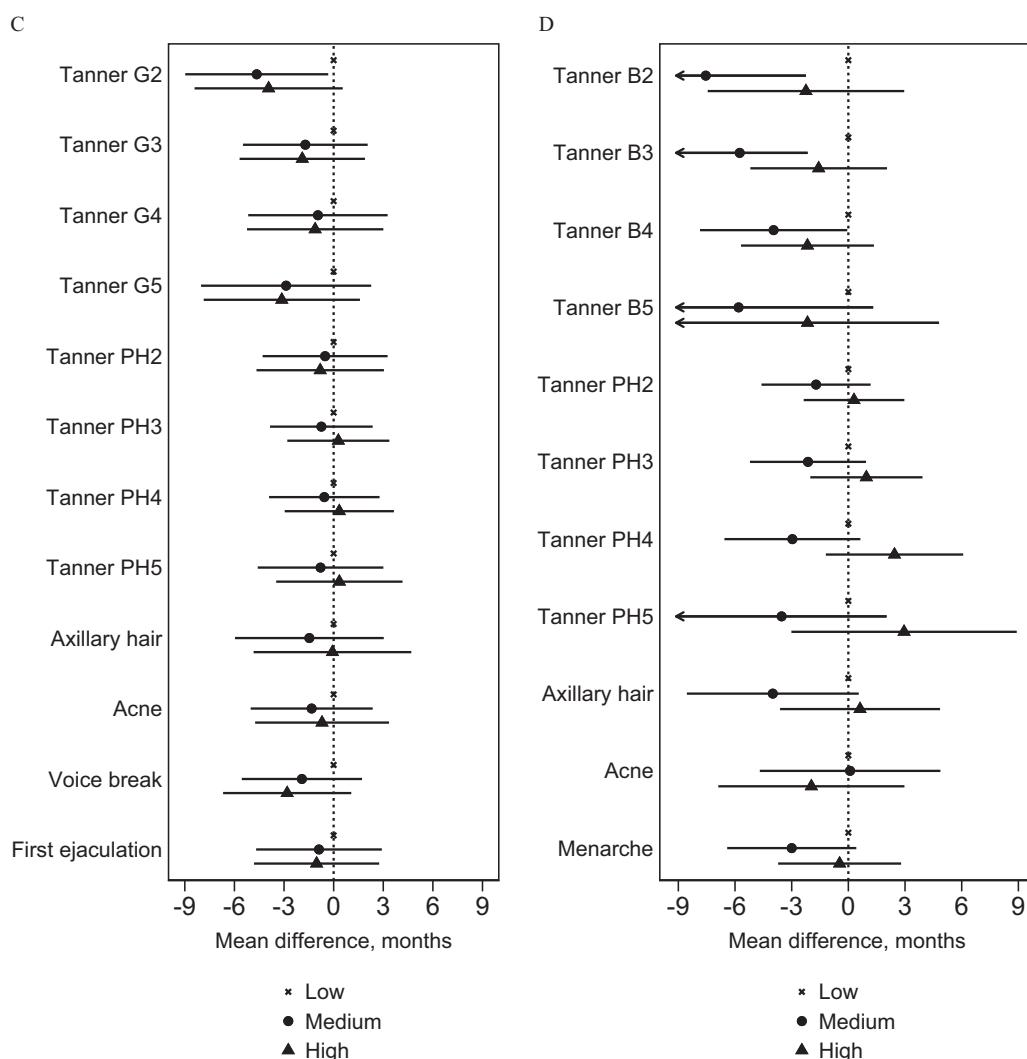
In boys, the mean age of onset was similar across PFOS tertiles for most of the outcomes, although the average ages of onset for Tanner stages of genital development and voice break were earlier among boys in the middle and highest tertiles, with similar differences from the lowest tertile (e.g., voice break:  $-2.7$  mo, 95% CI:  $-5.54$ ,  $-0.19$ , and  $-3.05$  mo, 95% CI:  $-5.77$ ,  $-0.34$  in the middle and highest tertiles, respectively) (Figure 2C; Table S3). In boys, mean ages of onset for all outcomes were lower with a doubling of prenatal PFOS, but CIs did not include the null only for Tanner genital Stage 2 ( $-3.10$  mo, 95% CI:  $-6.19$ ,  $0.00$ ) and voice break ( $-3.05$  mo, 95% CI:  $-5.77$ ,  $-0.34$ ) (Table 4). The middle ( $-1.67$  mo, 95% CI:  $-4.09$ ,  $0.75$ ) and highest PFOS tertile ( $-1.10$  mo, 95% CI:  $-3.60$ ,  $1.40$ ) of exposure were associated with lower average age of onset for the combined puberty indicator when compared to the lowest tertile (Table 5).

#### ***Perfluorohexane Sulfonate, Perfluoroheptane Sulfonate, Perfluorononanoic Acid, and Perfluorodecanoic Acid***

Sample 2 was smaller than sample 1 and included measurement of four other PFASs in addition to PFOA and PFOS.

For PFHxS, the highest vs. the lowest tertile of exposure was associated with earlier average age of onset for Tanner breast stages, axillary hair growth and menarche in girls, and earlier age of onset for all outcomes in boys except Tanner genital stage 2 (e.g., acne:  $-11.28$  mo, 95% CI:  $-19.26$ ,  $-3.31$  and voice break:  $-11.58$  mo, 95% CI:  $-20.23$ ,  $-2.93$ ) (Figure 3A–B; Table S4). The mean age differences in the middle vs. lowest tertile were primarily surrounded around the null in both sexes. A doubling of PFHxS exposure were associated with lower ages of onset for most of the male and female markers and generally lowest in boys (Table 6). The average age of onset for the combined indicator were lower in boys ( $-6.89$  mo, 95% CI:  $-12.57$ ,  $-1.20$ ) and in girls ( $-2.22$  mo, 95% CI:  $-8.37$ ,  $3.93$ ) from the highest PFHxS tertile vs. the lowest (Table 5).

PFHpS exposure in boys was associated with earlier mean age of onset for axillary hair growth, voice break, and acne in the highest tertile (Figure 3C; Table S4) and with a doubling of PFHpS for all outcomes except Tanner genital stages (Table 6) (e.g., voice break:  $-8.03$  mo, 95% CI:  $-19.34$ ,  $3.27$  in the highest tertile, and  $6.22$  mo, 95% CI:  $-11.90$ ,  $-0.54$  with a doubling of exposure). In girls, the middle vs. the lowest PFHpS tertile of



**Figure 2.** (Continued.)

was associated with earlier average age of onset for Tanner breast stages (e.g., Tanner breast stage 2:  $-9.05$  mo, 95% CI:  $-16.41$ ,  $-1.69$ ), acne, and menarche (Figure 3D; Table S4), while mean ages of onset for those in the highest PFHpS tertile were also lower or null. Departure from linearity was detected for 6 out of 11 female outcomes (Table 6). The mean difference for the combined puberty indicator was  $-1.20$  mo (95% CI:  $-6.76$ ,  $4.36$ ) and  $-4.48$  mo (95% CI:  $-9.88$ ,  $0.93$ ) in boys from the middle and highest tertiles, and  $-4.92$  mo (95% CI:  $-11.68$ ,  $1.85$ ) and  $-0.37$  mo (95% CI:  $-6.62$ ,  $5.87$ ) in girls from the middle and highest tertiles (Table 5).

Overall, PFNA tertiles of exposure were associated with later mean age of onset for male markers and earlier for female puberty markers (Figure 3E–F; Table S5). In boys, the mean age of onset for all outcomes, except first ejaculation, were higher in the middle PFNA tertile (e.g., Tanner genital stage 2:  $8.54$  mo, 95% CI:  $0.64$ ,  $16.44$ ), while the mean ages of onset in those from the highest tertile were overall closer to the null or even negative for acne, first ejaculation, and voice break ( $-7.90$  mo, 95% CI:  $-16.03$ ,  $0.24$ ). The associations with a doubling of PFNA exposure were inconsistent, and no departure from linearity was detected (Table 6). However, the mean age of onset for the combined indicator was higher in middle tertile ( $4.45$  mo, 95% CI:  $-1.30$ ,  $10.21$ ) than in the highest tertile ( $1.63$  mo,

95% CI:  $-3.82$ ,  $7.08$ ). In girls, mean ages of onset for all outcomes were negative in the middle and the highest tertiles (e.g., Tanner breast stage 2:  $-15.11$  mo, 95% CI:  $-23.00$ ,  $-7.22$  and  $-8.87$  mo, 95% CI:  $-16.01$ ,  $-1.72$  in the middle and highest tertiles, respectively), and no specific pattern of associations between those tertiles was detected (Figure 3F; Table S5). All associations were negative with a doubling of PFNA exposure (Table 6), and the mean age of onset for the combined indicator was  $-4.74$  mo (95% CI:  $-10.45$ ,  $0.96$ ) and  $-5.06$  mo (95% CI:  $-10.61$ ,  $0.48$ ) in the middle and highest tertiles, respectively (Table 5).

In boys, the mean ages of onset for all outcomes were lower in the middle vs. the lowest PFDA tertile but earlier in the highest tertile (Figure 3G; Table S5). In girls, the mean age of onset was negative for all outcomes in the middle PFDA tertile, while mean ages for those in the highest tertile (except Tanner Breast stage 2 and pubic hair stage 5) were closer to the null (Figure 3H; Table S5). The sex-specific patterns were also detected for the combined puberty indicator (Table 5) (boys:  $4.59$  mo, 95% CI:  $-0.93$ ,  $10.11$ , and  $-2.83$  mo, 95% CI:  $-8.43$ ,  $2.77$  in the middle and highest tertiles, respectively; and girls:  $-3.60$  mo (95% CI:  $-9.03$ ,  $1.83$ ) and  $0.02$  mo (95% CI:  $-6.64$ ,  $6.68$ ) in the middle and highest tertiles, respectively). However, no departure from linearity was detected in both boys and girls (Table 6).

**Table 4.** Estimated average differences in age when pubertal milestones were attained (in months) according to log2-transformed plasma PFOA and PFOS concentrations in first-trimester maternal samples from participants in the Puberty Cohort, Denmark (2017).

| Outcome                   | PFOA                                  |                       | PFOS                                  |                       |
|---------------------------|---------------------------------------|-----------------------|---------------------------------------|-----------------------|
|                           | Departure from linearity <sup>b</sup> |                       | Departure from linearity <sup>b</sup> |                       |
|                           | $\beta^a$ (95% CI)                    | Test: <i>p</i> -value | $\beta^a$ (95% CI)                    | Test: <i>p</i> -value |
| Boys ( <i>n</i> = 565)    | —                                     | —                     | —                                     | —                     |
| Tanner stages: genitals   | —                                     | —                     | —                                     | —                     |
| Stage 2                   | −1.93 (−4.65, 0.80)                   | 0.57                  | −3.10 (−6.19, 0.00)                   | 0.66                  |
| Stage 3                   | −1.14 (−3.40, 1.12)                   | 0.57                  | −2.47 (−5.01, 0.07)                   | 0.77                  |
| Stage 4                   | −1.71 (−4.26, 0.84)                   | 0.24                  | −1.79 (−4.72, 1.13)                   | 0.60                  |
| Stage 5                   | −1.43 (−4.72, 1.86)                   | 0.37                  | −2.51 (−5.95, 0.93)                   | 0.19                  |
| Tanner stages: pubic hair | —                                     | —                     | —                                     | —                     |
| Stage 2                   | 0.36 (−2.00, 2.73)                    | 0.92                  | −1.50 (−4.08, 1.08)                   | 0.55                  |
| Stage 3                   | −0.22 (−2.19, 1.75)                   | 0.83                  | −0.34 (−2.53, 1.84)                   | 0.94                  |
| Stage 4                   | −0.12 (−2.19, 1.94)                   | 0.23                  | −0.99 (−3.22, 1.25)                   | 0.82                  |
| Stage 5                   | 0.74 (−1.83, 3.31)                    | 0.43                  | −0.60 (−3.39, 2.19)                   | 0.13                  |
| Axillary hair             | 1.09 (−1.89, 4.08)                    | 0.30                  | −0.37 (−3.58, 2.84)                   | 0.70                  |
| Acne                      | −1.06 (−3.62, 1.49)                   | 0.58                  | −1.52 (−4.52, 1.48)                   | 0.39                  |
| Voice break               | −2.71 (−5.24, −0.19)                  | 0.15                  | −3.05 (−5.77, −0.34)                  | 0.80                  |
| First ejaculation         | −1.22 (−3.83, 1.38)                   | 0.23                  | −0.62 (−3.36, 2.12)                   | 0.88                  |
| Girls ( <i>n</i> = 555)   | —                                     | —                     | —                                     | —                     |
| Tanner stages: breast     | —                                     | —                     | —                                     | —                     |
| Stage 2                   | −4.07 (−8.12, −0.02)                  | 0.08                  | −3.08 (−7.15, 0.98)                   | 0.10                  |
| Stage 3                   | −1.04 (−3.61, 1.53)                   | 0.54                  | −1.75 (−4.43, 0.93)                   | 0.29                  |
| Stage 4                   | −0.69 (−3.18, 1.80)                   | 0.36                  | −2.20 (−4.68, 0.29)                   | 0.03                  |
| Stage 5                   | −1.37 (−6.14, 3.40)                   | 0.77                  | −3.01 (−7.96, 1.95)                   | 0.03                  |
| Tanner stages: pubic hair | —                                     | —                     | —                                     | —                     |
| Stage 2                   | 0.30 (−1.68, 2.27)                    | 0.35                  | 0.51 (−1.62, 2.64)                    | 0.10                  |
| Stage 3                   | −0.05 (−2.03, 1.92)                   | 0.05                  | 0.07 (−2.05, 2.19)                    | 0.00                  |
| Stage 4                   | 0.46 (−2.12, 3.03)                    | 0.05                  | 1.11 (−1.58, 3.79)                    | 0.00                  |
| Stage 5                   | 3.05 (−0.94, 7.04)                    | 0.01                  | 1.81 (−2.42, 6.04)                    | 0.20                  |
| Axillary hair             | −1.49 (−4.56, 1.58)                   | 0.74                  | 0.50 (−2.79, 3.79)                    | 0.02                  |
| Acne                      | −5.16 (−8.50, −1.82)                  | 0.58                  | −1.73 (−5.24, 1.77)                   | 0.17                  |
| Menarche                  | −1.09 (−3.25, 1.07)                   | 0.83                  | −0.68 (−3.13, 1.77)                   | 0.00                  |

Note: —, data not available; CI, confidence interval; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid. Adjusted for highest social class of parents, maternal age at menarche, maternal age at delivery, parity, prepregnancy body mass index, and daily number of cigarettes smoked in first trimester. Results presented as a meta-analysis of sample 1 and sample 2. Sample-specific estimates can be found in Tables S1 and S2.

<sup>a</sup>The  $\beta$  coefficient corresponds to the mean monthly difference per doubling of predictor variable.

<sup>b</sup>Continuous log2-transformed concentrations modeled as a second-order polynomial to test for quadratic departure from linearity. *p*-Values (two-sided) are presented.

**Table 5.** Estimated average differences in the age when a combined sex-specific puberty indicator was attained (in months) according to tertiles of plasma PFAS concentrations in first-trimester maternal samples from participants in the Puberty Cohort, Denmark (2017).

| Type of PFAS and tertile of exposure               | Combined puberty indicator <sup>a</sup> |                                      |
|----------------------------------------------------|-----------------------------------------|--------------------------------------|
|                                                    | Boys                                    | Girls                                |
|                                                    | Age difference (95% CI) <sup>b</sup>    | Age difference (95% CI) <sup>b</sup> |
| PFOA <sup>c</sup> ( <i>n</i> = 565 boys/555 girls) | —                                       | —                                    |
| Medium                                             | 0.71 (−1.88, 3.29)                      | −0.64 (−3.43, 2.16)                  |
| High                                               | −0.08 (−2.56, 2.40)                     | −0.39 (−3.25, 2.47)                  |
| PFOS <sup>c</sup> ( <i>n</i> = 565 boys/555 girls) | —                                       | —                                    |
| Medium                                             | −1.67 (−4.09, 0.75)                     | −3.73 (−6.59, −0.87)                 |
| High                                               | −1.10 (−3.60, 1.40)                     | −0.17 (−2.83, 2.49)                  |
| PFHxS ( <i>n</i> = 227 boys/206 girls)             | —                                       | —                                    |
| Medium                                             | 0.17 (−5.48, 5.82)                      | −0.21 (−6.84, 6.41)                  |
| High                                               | −6.89 (−12.57, −1.20)                   | −2.22 (−8.37, 3.93)                  |
| PFHpS ( <i>n</i> = 227 boys/206 girls)             | —                                       | —                                    |
| Medium                                             | −1.20 (−6.76, 4.36)                     | −4.92 (−11.68, 1.85)                 |
| High                                               | −4.48 (−9.88, 0.93)                     | −0.37 (−6.62, 5.87)                  |
| PFNA ( <i>n</i> = 227 boys/206 girls)              | —                                       | —                                    |
| Medium                                             | 4.45 (−1.30, 10.21)                     | −4.74 (−10.45, 0.96)                 |
| High                                               | 1.63 (−3.82, 7.08)                      | −5.06 (−10.61, 0.48)                 |
| PFDA ( <i>n</i> = 227 boys/206 girls)              | —                                       | —                                    |
| Medium                                             | 4.59 (−0.93, 10.11)                     | −3.60 (−9.03, 1.83)                  |
| High                                               | −2.83 (−8.43, 2.77)                     | 0.02 (−6.64, 6.68)                   |

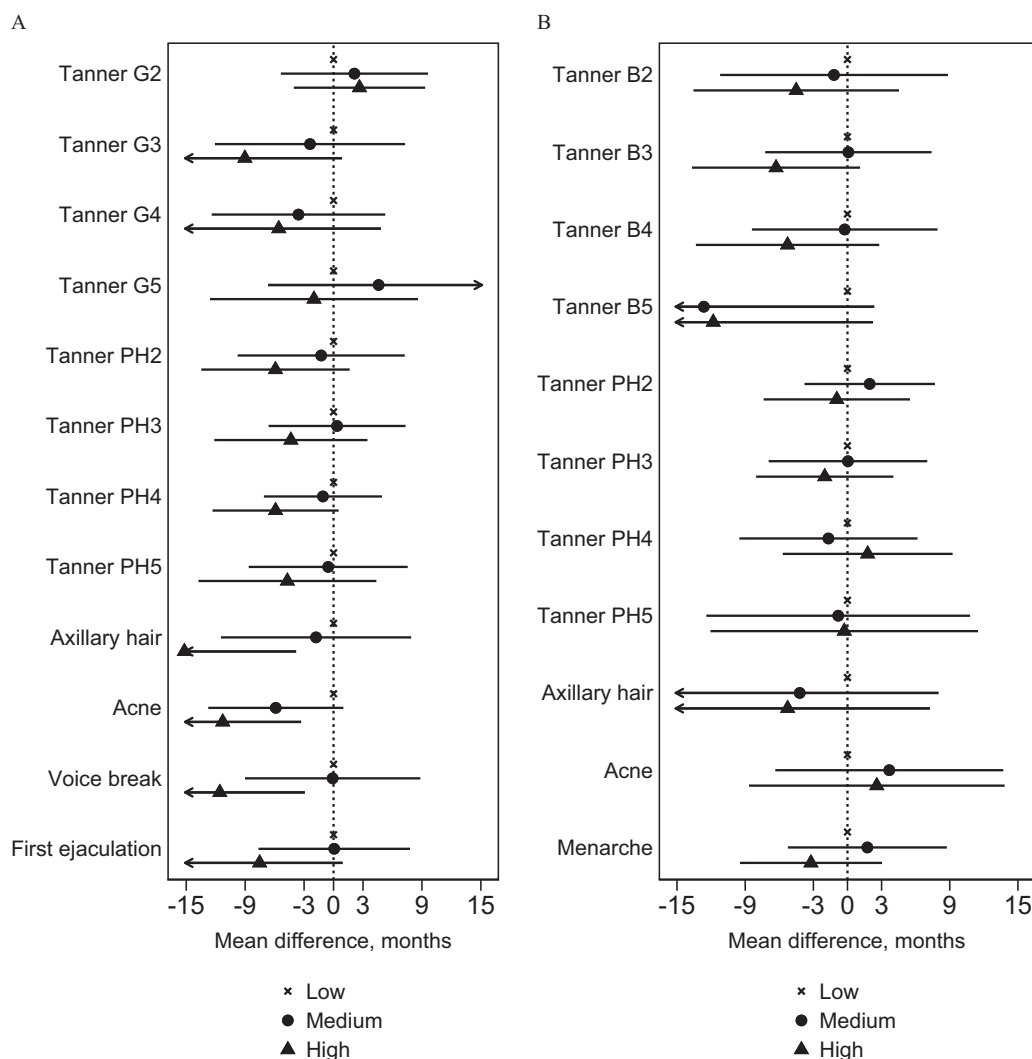
Note: —, data not available; CI, Confidence interval; PFDA, perfluorodecanoic acid; PFHpS, perfluoroheptane sulfonate; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid.

<sup>a</sup>All sex-specific pubertal indicators combined in one model.

<sup>b</sup>Adjusted mean monthly differences estimated by the Huber-White robust variance estimation using boys and girls from the lowest tertile of exposure as reference.

<sup>c</sup>The presented results for PFOA and PFOS are from a meta-analysis of sample 1 and sample 2.





**Figure 3.** Estimated average difference (with 95% confidence intervals) in age (months) when pubertal milestones were attained according to tertiles of plasma PFHxS, PFHpS, PFNA, and PFDA concentrations in first-trimester maternal samples. All estimates are adjusted for highest social class of parents, maternal age at menarche, maternal age at delivery, parity, prepregnancy body mass index, and maternal smoking during first trimester. (A) PFHxS in boys. (B) PFHxS in girls. (C) PFHpS in boys. (D) PFHpS in girls. (E) PFNA in boys. (F) PFNA in girls. (G) PFDA in boys. (H) PFDA in girls. See Tables S4 and S5 for corresponding numeric data and Table 2 for tertile cutoff points. Note: PFDA, perfluorodecanoic acid; PFHpS, perfluoroheptane sulfonate; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoic acid; Tanner B2-5, Tanner breast stage 2-5; Tanner G2-5, Tanner genital stage 2-5; Tanner PH2-5, Tanner pubic hair stage 2-5.

## Discussion

In summary, prenatal exposure to PFOS, PFHxS, PFHpS, PFNA, and PFDA was associated with lower average age at onset for the individual puberty milestones and a combined puberty indicator in girls. However, for PFOS, PFHpS, and PFDA in girls, the mean ages of onset for the puberty milestones and the combined puberty indicator were lower in the middle- than in the highest-exposure tertiles. In boys, we found both positive and negative associations with prenatal exposure to different types of PFASs. PFHxS and PFHpS were associated with lower mean ages of timing of puberty, while PFNA and PFDA were associated with higher mean ages of timing of puberty. However, for PFNA and PFDA in boys, the mean ages at onset for some of the puberty milestones and for the combined puberty indicator were higher in the middle than in the highest exposure tertile.

The hypothalamic–pituitary–gonadal axis (HPG axis) plays a crucial role in the timing of puberty and may be particularly prone to exogenous exposures in two developmental phases.

First, the HPG axis and the gonads are formed in the first trimester and are active throughout the pregnancy. Secondly, in the first 6–9 mo of life, the HPG axis remains active, which is known as a phenomenon called the mini puberty (Kuirri-Hänninen et al. 2014). Hereafter, the HPG axis enters the silent juvenile phase until it reactivates in the prepubertal phase and initiates puberty. This study cannot exclude that the observed associations reflect exposure to PFASs during the mini puberty through breastfeeding (Haug et al. 2011; Verner et al. 2016) or in other childhood exposure windows rather than prenatal exposure or the combined exposure in multiple periods. However, first-trimester exposure to other environmental agents with hormone-disrupting potential has been associated with other negative reproductive outcomes in the offspring, suggesting that the first trimester may be an important programming window (Pryor et al. 2000).

Since this is one of the first studies to explore the associations between prenatal exposure to PFASs and pubertal development, these findings need to be replicated. As discussed by Vandenberg et al. (2012), there is a considerable body of experimental data

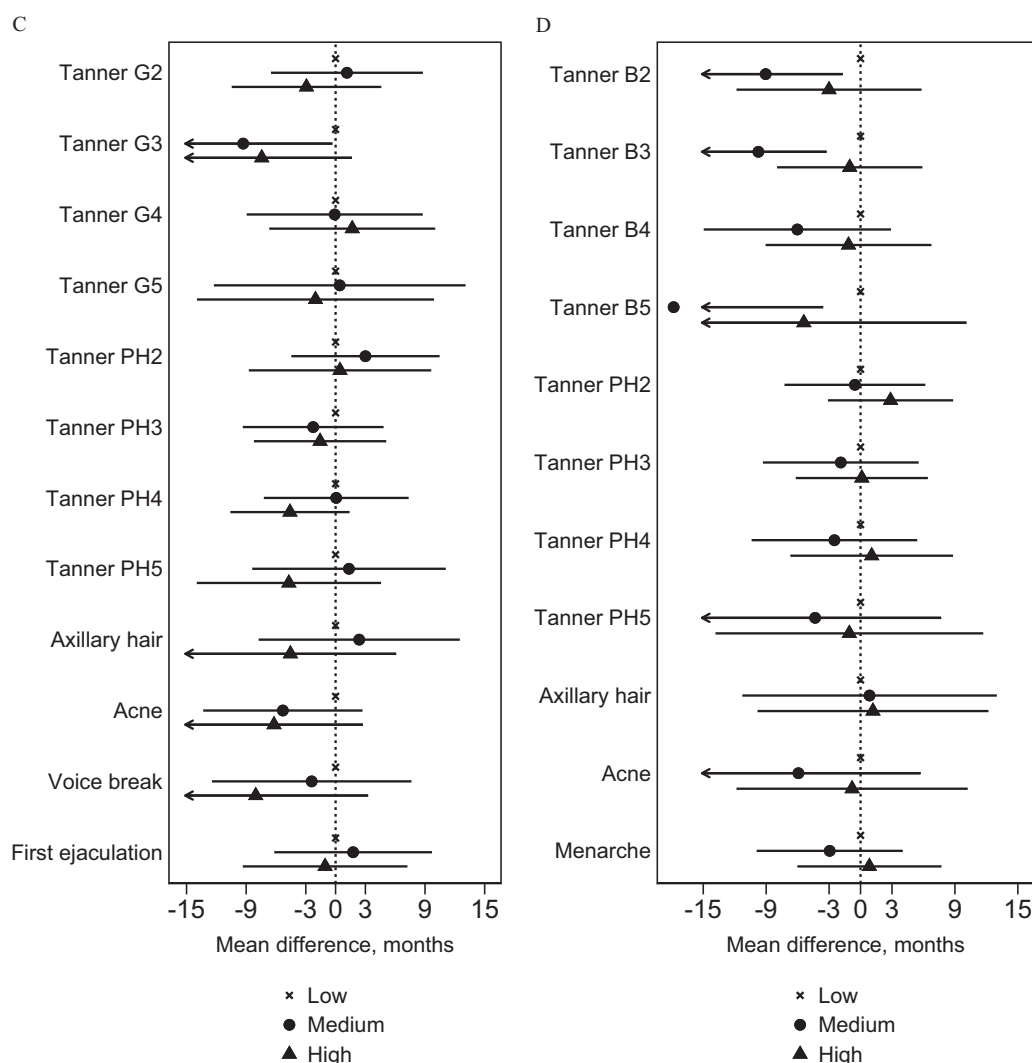


Figure 3. (Continued.)

to support the biological plausibility of nonmonotonic effects of exposure to endocrine-disrupting chemicals (EDCs) in the environment, such as PFAS. Despite biological plausibility, few epidemiological studies have presented nonmonotonic associations between EDCs including PFASs and health outcomes, which might be explained by the different exposure levels and mixtures across study populations. Other sources of bias and chance findings should also be considered. High levels of prenatal PFASs have been shown to affect fetal loss and adverse birth outcomes (Liew et al. 2015; Meng et al. 2018); thus, an underestimation of the association for the highly exposed group is possible if the exposed fetuses were less likely to be included in the follow-up (either due to death or nonparticipation due to health status). However, the overall direction of the associations was quite consistent in our data, suggesting lower mean age at onset for several pubertal milestones in all studies PFASs except PFDA and PFNA in boys. Still, we warrant for cautious interpretation and further evaluations on the possible nonmonotonic exposure response.

Few previously published studies have investigated the potential long-term effects of gestational PFAS exposure on reproductive health, including markers of pubertal development. In a Danish study of 343 females from a different cohort, Kristensen et al. (2013) associated PFOA exposure with delayed age at

menarche, but reported no changes in levels of reproductive hormones or menstrual cycle characteristics. In contrast to our findings, no associations with prenatal PFOS exposure were reported in that study. Examining 169 young males from the same pregnancy cohort as Kristensen et al. (2013), Vested et al. (2013) found an association between impaired semen quality and higher levels of luteinizing and follicle-stimulating hormone with *in utero* exposure to PFOA. Higher levels of luteinizing and follicle-stimulating hormone may potentially lead to earlier pubertal development. On the other hand, PFOS was not associated with the examined markers of male reproductive health in the study by Vested et al. (2013).

Two studies have used data from the ALSPAC to examine the association between exposure to PFAS and reproductive health in females. In a nested case-control study, early puberty before the age of 11.5 y was not associated with increased odds of higher PFASs levels (Christensen et al. 2011). However, Maisonet et al. (2015) found association between *in utero* exposure to PFOA, PFOS, and PFHxS and increasing levels of testosterone in a subsequent study of 72 adolescent girls from ALSPAC, suggesting a potential mechanism by which PFASs may induce early puberty development in girls. Beside these findings, two cross-sectional studies of adolescents from the C8 Health Project found associations between increasing serum levels of PFASs and

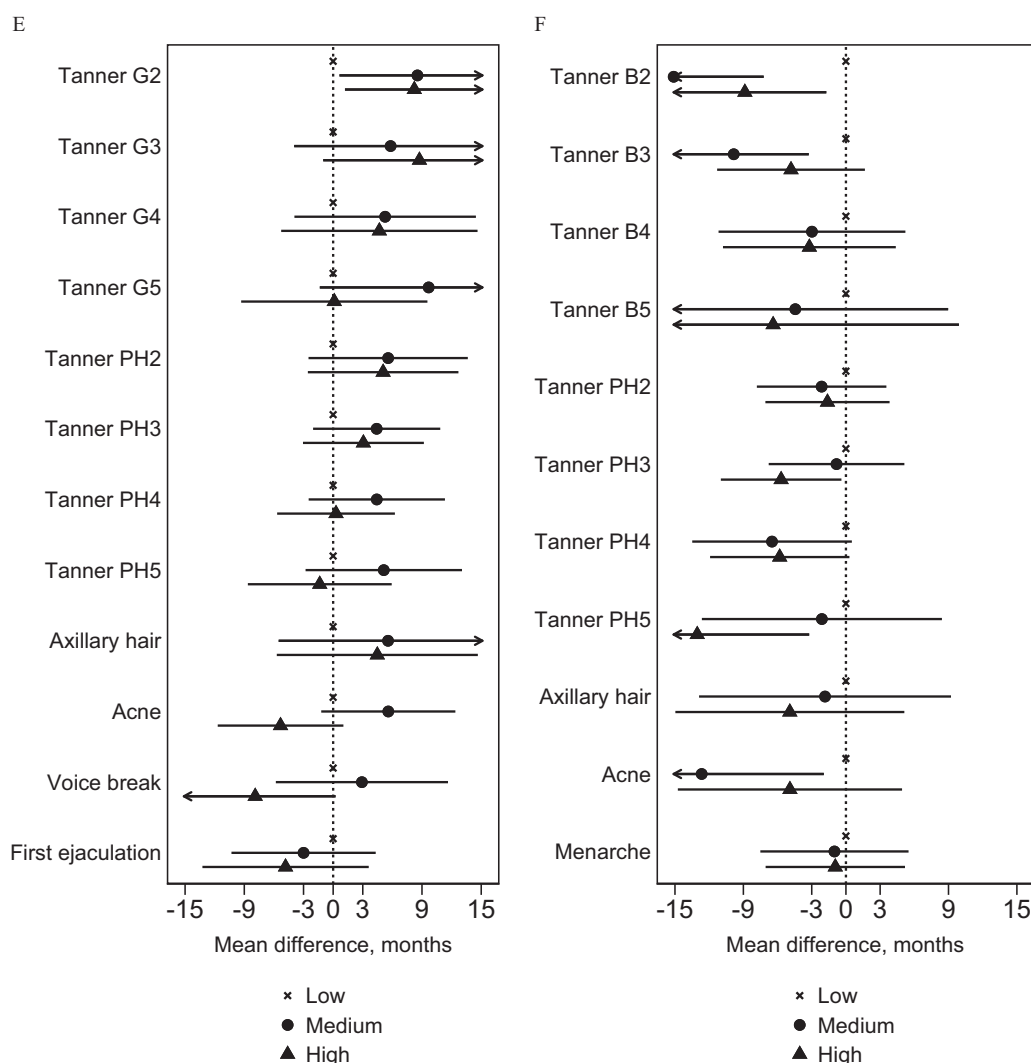


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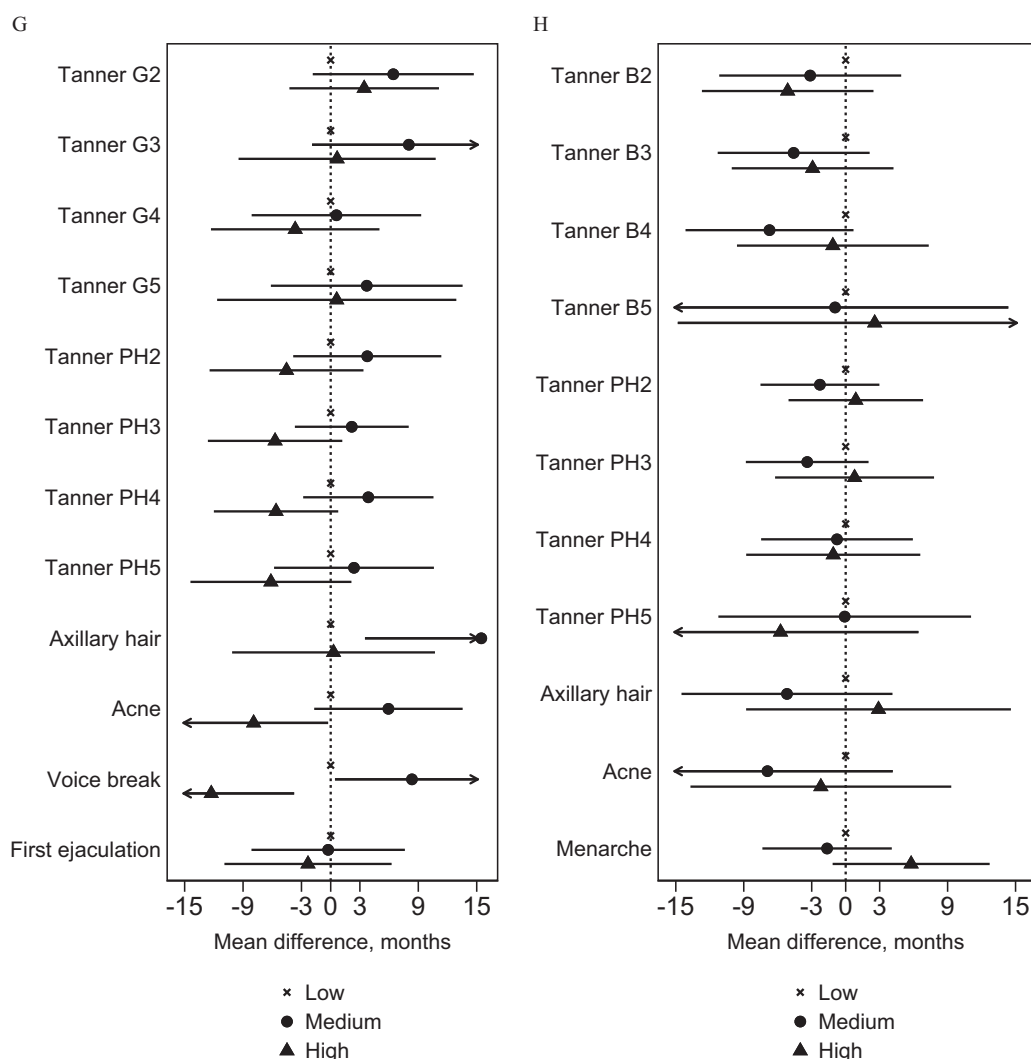
different markers of later mean onset of puberty (Lopez-Espinosa et al. 2011) as well as lower levels of sex hormones in children of both genders (Lopez-Espinosa et al. 2016). However, these findings are not directly comparable to ours, as PFAS measurements in that study were obtained during late childhood or early adolescence.

A potential mechanism by which prenatal exposure to PFAS may lead to earlier pubertal development relies on emerging epidemiologic evidence associating pre- and postnatal exposure to PFASs with increased risk of childhood obesity in both sexes (Braun 2017). As increasing prepubertal BMI has been associated with earlier pubertal development (Aksglaede et al. 2009), any potential effect of prenatal PFAS exposure on timing of puberty, as suggested in this study, may have been partly mediated through changes in prepubertal BMI. However, in our study samples, prepubertal BMI was evenly distributed across tertiles of PFOA and PFOS exposure (Table S6), which is in line with a previous paper on prenatal to PFOA and PFOS and BMI at age 7 in the DNBC (Andersen et al. 2013).

In addition, several *in vivo* and *in vitro* studies have examined the potential endocrine-disrupting properties of PFASs. Kjeldsen and Bonefeld-Jørgensen (2013) suggested that PFOA, PFOS, and PFHxS have the ability to act as an estrogen receptor agonist, whereas PFHxS, PFOS, PFOA, PFNA, and PFDA antagonized

androgen receptor transactivity. These findings support animal studies that report decreased levels of testosterone and increased levels of estrogen in serum from rats exposed to PFASs (Lau et al. 2007). Although speculative, these various endocrine properties may explain why the associations differed among the PFASs studied and contribute to the potential sex- and puberty marker-specific associations observed. Potentially, increasing estrogen receptor activity following prenatal PFAS exposure may induce earlier female pubertal development in itself or by interfering with the reactivation of the HPG axis during sexual maturation. If PFASs also act as androgen receptor antagonists, we would expect later rather than earlier male pubertal development, as observed in our study.

Our study included relatively large samples of both boys and girls, especially for the PFOS and PFOA analyses. We were able to evaluate the possible associations with other PFASs than PFOA and PFOS, although these results were based on a smaller sample, and estimates had a high, large statistical uncertainty. Further, exposure assessment was conducted previously, blinded to the outcomes of interest. For PFOA and PFOS, blood sampling took place in a period with high exposure levels, thereby allowing us to explore the associations in a group of children with large exposure contrasts. Our study samples stem from a large nationwide cohort with a long follow-up, holding detailed and longitudinally



**Figure 3.** (Continued.)

collected information on important confounders such as socioeconomic status and maternal age at menarche. We used longitudinally collected multiple (with 6-mo intervals throughout puberty) measurements of pubertal development covering various aspects of sexual maturation. Different neuroendocrine pathways regulate the processes leading to the development of specific secondary sexual characteristics (Grumbach 2002). When we analyzed the specific puberty milestones individually, we attempted to capture potential programming effects of specific features of pubertal development, such as the association between prenatal PFOS exposure and Tanner stages of breast development, but not the remaining markers. However, puberty covers a continuum of developmental processes, and age at onset of pubertal milestones may be correlated within individuals. To account for any correlation structures, we analyzed all puberty markers in one model for each PFAS stratified by sex. The results for the combined puberty indicators supported the findings from the individual marker-models.

We used a single measure of PFAS concentrations in maternal blood collected in early pregnancy as a surrogate measure of fetal exposure. This may create some exposure misclassification, since it remains unknown when the main transfer of PFASs occurs and whether the transfer is faster/slower at certain periods throughout gestation, and each PFAS has different

capabilities of transplacental transfer (Apelberg et al. 2007; Fei et al. 2007). However, it has previously been shown that PFAS measures are very highly correlated in repeated maternal pregnancy samples and cord blood samples in the DNBC (Fei et al. 2007). Thus, any misclassification of fetal exposure to PFAS is likely to be minor and possibly nondifferential, considering the long-term health outcomes.

An important limitation is the late age at entry in the Puberty Cohort. Thus, large proportions of participants had already experienced early indicators of puberty (Tanner Gonadal stage 2+ = 65 %, Tanner pubic hair stage 2+ = 52 %, Tanner breast stage 2+ = 85 %, and Tanner pubic hair stage 2+ = 54 %). However, given that the assumption of normally distributed residuals are true, we should yield valid estimates for all indicators of pubertal development. Information on pubertal development was based on self-assessment and showed some degree of misclassification when we compared the self-reported information with a clinical examination in a recent validation study of 200 adolescents from the Puberty Cohort (Ernst et al. 2018). However, we have no reason to expect that this misclassification depends on the level of PFAS exposure.

As the mechanistic endocrine effects of different PFASs may depend on their individual biochemical properties (Buck et al. 2011), we chose to examine associations by individual PFAS.



**Table 6.** Estimated average differences in age when pubertal milestones were attained (in months) according to log2-transformed plasma PFHxS, PFHpS, PFNA, and PFDA concentrations in first-trimester maternal samples from participants in the Puberty Cohort, Denmark (2017).

|                               | PFHxS                 |                                                                   |  | PFHpS                 |                                                                   |  | PFNA                  |                                                                   |  | PFDA                  |                                                                   |  |
|-------------------------------|-----------------------|-------------------------------------------------------------------|--|-----------------------|-------------------------------------------------------------------|--|-----------------------|-------------------------------------------------------------------|--|-----------------------|-------------------------------------------------------------------|--|
|                               | $\beta^a$<br>(95% CI) | Departure from<br>linearity <sup>b</sup><br>Test: <i>p</i> -value |  | $\beta^a$<br>(95% CI) | Departure from<br>linearity <sup>b</sup><br>Test: <i>p</i> -value |  | $\beta^a$<br>(95% CI) | Departure from<br>linearity <sup>b</sup><br>Test: <i>p</i> -value |  | $\beta^a$<br>(95% CI) | Departure from<br>linearity <sup>b</sup><br>Test: <i>p</i> -value |  |
| <b>Boys (<i>n</i> = 227)</b>  |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |
| Tanner stages: genitals       |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |
| Stage 2                       | 1.22 (−2.73, 5.17)    | 0.71                                                              |  | −0.99 (−5.12, 3.13)   | 0.32                                                              |  | 2.34 (−3.10, 7.78)    | 0.63                                                              |  | 1.88 (−2.83, 6.59)    | 0.67                                                              |  |
| Stage 3                       | −6.93 (−12.52, −1.34) | 0.62                                                              |  | −5.24 (−9.55, −0.92)  | 0.30                                                              |  | 3.77 (−3.42, 10.96)   | 0.11                                                              |  | 1.71 (−4.65, 8.06)    | 0.07                                                              |  |
| Stage 4                       | −5.11 (−10.31, 0.08)  | 0.02                                                              |  | −0.94 (−5.15, 3.27)   | 0.04                                                              |  | 1.92 (−5.28, 9.12)    | 0.26                                                              |  | 0.65 (−5.23, 6.54)    | 0.01                                                              |  |
| Stage 5                       | −4.13 (−11.11, 2.84)  | 0.05                                                              |  | −0.28 (−7.28, 6.71)   | 0.07                                                              |  | 1.74 (−6.77, 10.24)   | 0.31                                                              |  | 3.71 (−3.74, 11.16)   | 0.01                                                              |  |
| Tanner stages: pubic hair     |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |
| Stage 2                       | −3.33 (−7.45, 0.80)   | 0.71                                                              |  | −2.57 (−6.39, 1.25)   | 0.37                                                              |  | 1.35 (−4.85, 7.54)    | 0.49                                                              |  | −1.94 (−7.93, 4.06)   | 0.28                                                              |  |
| Stage 3                       | −3.24 (−7.23, 0.75)   | 0.02                                                              |  | −2.44 (−6.31, 1.44)   | 0.24                                                              |  | 0.68 (−4.24, 5.61)    | 0.13                                                              |  | −1.27 (−5.51, 2.97)   | 0.01                                                              |  |
| Stage 4                       | −4.46 (−7.99, −0.92)  | 0.03                                                              |  | −3.55 (−6.62, −0.49)  | 0.52                                                              |  | −0.74 (−5.39, 3.90)   | 0.24                                                              |  | −2.12 (−6.36, 2.12)   | 0.05                                                              |  |
| Stage 5                       | −5.66 (−11.24, −0.08) | 0.14                                                              |  | −2.62 (−8.36, 3.11)   | 0.29                                                              |  | −1.82 (−7.83, 4.19)   | 0.41                                                              |  | −2.69 (−7.95, 2.57)   | 0.12                                                              |  |
| Axillary hair                 | −8.35 (−14.50, −2.20) | 0.11                                                              |  | −4.10 (−9.63, 1.43)   | 0.07                                                              |  | 1.19 (−6.50, 8.88)    | 0.64                                                              |  | 0.68 (−5.35, 6.70)    | 0.98                                                              |  |
| Acne                          | −7.41 (−12.03, −2.78) | 0.29                                                              |  | −4.60 (−9.64, 0.44)   | 0.87                                                              |  | −8.15 (−13.00, −3.31) | 0.09                                                              |  | −5.34 (−9.94, −0.73)  | 0.37                                                              |  |
| Voice break                   | −7.04 (−12.44, −1.63) | 0.42                                                              |  | −6.22 (−11.90, −0.54) | 0.94                                                              |  | −8.25 (−14.44, −2.06) | 0.14                                                              |  | −7.50 (−14.17, −0.83) | 0.68                                                              |  |
| First ejaculation             | −4.90 (−9.05, −0.75)  | 0.87                                                              |  | −3.39 (−8.82, 2.05)   | 0.74                                                              |  | −3.83 (−9.11, 1.45)   | 0.61                                                              |  | −4.25 (−10.41, 1.91)  | 0.90                                                              |  |
| <b>Girls (<i>n</i> = 206)</b> |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |
| Tanner stages: breast         |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |
| Stage 2                       | −0.53 (−6.11, 5.04)   | 0.13                                                              |  | −3.56 (−9.06, 1.94)   | 0.78                                                              |  | −8.78 (−16.55, −1.02) | 0.48                                                              |  | −4.21 (−9.86, 1.43)   | 0.84                                                              |  |
| Stage 3                       | −3.21 (−7.51, 1.10)   | 0.92                                                              |  | −1.94 (−6.19, 2.30)   | 0.09                                                              |  | −4.92 (−10.70, 0.87)  | 0.50                                                              |  | −2.19 (−7.37, 2.99)   | 0.72                                                              |  |
| Stage 4                       | −1.90 (−7.31, 3.50)   | 0.25                                                              |  | −2.33 (−6.65, 2.00)   | 0.02                                                              |  | −3.78 (−10.12, 2.56)  | 0.24                                                              |  | −1.02 (−6.72, 4.68)   | 0.92                                                              |  |
| Stage 5                       | −5.83 (−16.98, 5.33)  | 0.78                                                              |  | −2.95 (−11.75, 5.85)  | 0.31                                                              |  | −3.94 (−16.37, 8.49)  | 0.53                                                              |  | 1.11 (−11.55, 13.78)  | 0.91                                                              |  |
| Tanner stages: pubic hair     |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |                       |                                                                   |  |
| Stage 2                       | 1.55 (−2.36, 5.45)    | 0.81                                                              |  | 0.25 (−3.60, 4.10)    | 0.03                                                              |  | −2.40 (−7.03, 2.22)   | 0.76                                                              |  | 1.26 (−3.02, 5.53)    | 0.85                                                              |  |
| Stage 3                       | 0.50 (−3.47, 4.48)    | 0.81                                                              |  | −1.50 (−4.74, 1.73)   | 0.05                                                              |  | −4.10 (−8.56, 0.37)   | 0.05                                                              |  | 0.25 (−3.97, 4.47)    | 0.65                                                              |  |
| Stage 4                       | 1.74 (−3.64, 7.12)    | 0.74                                                              |  | −0.08 (−4.59, 4.43)   | 0.00                                                              |  | −5.37 (−10.72, −0.02) | 0.19                                                              |  | −0.89 (−6.31, 4.53)   | 0.93                                                              |  |
| Stage 5                       | 1.15 (−7.49, 9.78)    | 0.71                                                              |  | −0.77 (−8.21, 6.66)   | 0.09                                                              |  | −9.51 (−17.68, −1.35) | 0.05                                                              |  | −4.04 (−12.41, 4.32)  | 0.86                                                              |  |
| Axillary hair                 | −1.22 (−8.33, 5.90)   | 0.58                                                              |  | 1.13 (−5.43, 7.70)    | 0.57                                                              |  | −4.23 (−12.13, 3.67)  | 0.15                                                              |  | 3.67 (−3.50, 10.85)   | 0.33                                                              |  |
| Acne                          | 1.01 (−7.42, 9.44)    | 0.32                                                              |  | −2.44 (−9.87, 5.00)   | 0.03                                                              |  | −5.62 (−15.44, 4.21)  | 0.29                                                              |  | −0.90 (−10.05, 8.25)  | 0.03                                                              |  |
| Menarche                      | −1.12 (−5.94, 3.70)   | 0.18                                                              |  | −0.61 (−5.06, 3.83)   | 0.03                                                              |  | −1.86 (−7.51, 3.79)   | 0.17                                                              |  | 3.52 (−1.22, 8.25)    | 0.84                                                              |  |

Note: —, data not available; CI, confidence interval; PFDA, perfluorodecanoic acid; PFHpS, perfluorohexane sulfonate; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoic acid. Adjusted for highest social class of parents, maternal age at menarche, maternal age at delivery, parity, pre-pregnancy body mass index, and daily number of cigarettes smoked in first trimester.

<sup>a</sup>The  $\beta$  coefficient corresponds to the mean monthly difference per doubling of predictor variable.

<sup>b</sup>Continuous log2-transformed concentrations modeled as a second-order polynomial to test for quadratic departure from linearity. *p*-Values (two-sided) are presented.

These associations may be confounded by simultaneous exposure to other PFASs. Some advanced methods for mixture analyses have been proposed, but they come with their own limitations. One relies on standardization of exposure values (Govarts et al. 2016), which makes it difficult to compare results across studies from populations exposed to different levels of PFASs. Another uses structural equation models to derive a latent construct for PFAS coexposures (Valvi et al. 2017). The variation in the latent construct may reflect the sources of exposure shared by the different PFAS rather than the casual effect of the total exposure from multiple PFASs.

## Conclusions

In this longitudinal study in Denmark, we found that prenatal exposure to several types of PFASs was associated with altered timing of pubertal development. The magnitude and pattern of the associations varied with the child's sex, and some nonmonotonic responses between prenatal PFAS exposure and specific puberty indicators were observed. These findings are novel, and replication is needed. Due to the ubiquitous gestational exposure to PFASs, potential fetal programming of pubertal development has public health relevance and should warrant further investigation.

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