Human Reproduction, Vol.29, No.2 pp. 359–367, 2014
Advanced Access publication on October 25, 2013 doi:10.1093/humrep/det390

human reproduction

ORIGINAL ARTICLE ***Reproductive epidemiology***

Menstrual cycle characteristics in fertile women from Greenland, Poland and Ukraine exposed to perfluorinated chemicals: a cross-sectional study

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Submitted on April 22, 2013; resubmitted on September 14, 2013; accepted on September 19, 2013

STUDY QUESTION: Does perfluorooctane sulfonate (PFOS) and perfluorooctanate (PFOA) exposure disrupt the menstrual cyclicity?

SUMMARY ANSWER: The female reproductive system may be sensitive to PFOA exposure, with longer menstrual cycle length at higher exposure.

WHAT IS KNOWN ALREADY: PFOS and PFOA are persistent man-made chemicals. Experimental animal studies suggest they are reproductive toxicants but epidemiological findings are inconsistent.

STUDY DESIGN, SIZE, DURATION: A cross-sectional study including 1623 pregnant women from the INUENDO cohort enrolled during antenatal care visits between June 2002 and May 2004 in Greenland, Poland and Ukraine.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Information on menstrual cycle characteristics was obtained by questionnaires together with a blood sample from each pregnant woman. Serum concentrations of PFOS and PFOA were measured by liquid chromatography tandem mass spectrometry. Multiple imputations were performed to account for missing data. The association between PFOS/PFOA and menstrual cycle length (short cycle: $\leq$24 days, long cycle: $\geq$32 days) and irregularities ($\geq$7 days in difference between cycles) was analyzed using logistic regression with tertiles of exposure. Estimates are given as adjusted odds ratios (ORs) with 95% confidence intervals (CIs).

MAIN RESULTS AND THE ROLE OF CHANCE: Higher exposure levels of PFOA were associated with longer menstrual cycles in pooled estimates of all three countries. Compared with women in the lowest exposure tertile, the adjusted OR of long cycles was 1.8 (95% CI: 1.0; 3.3) among women in the highest tertile of PFOA exposure. No significant associations were observed between PFOS exposure and menstrual cycle characteristics. However, we observed a tendency toward more irregular cycles with higher exposure to PFOS [OR 1.7 (95% CI: 0.8; 3.5)]. The overall response rate was 45.3% with considerable variation between countries (91.3% in Greenland, 69.1% in Poland and 26.3% in Ukraine).

LIMITATIONS, REASONS FOR CAUTION: Possible limitations in our study include varying participation rates across countries; a selected study group overrepresenting the most fertile part of the population; retrospective information on menstrual cycle characteristics; the determination of cut-points for all three outcome variables; and lacking information on some determinants of menstrual cycle characteristics, such as stress, physical activity, chronic diseases and gynecological disorders, thus confounding cannot be excluded.

WIDER IMPLICATIONS OF THE FINDINGS: The generalizability of the study results is restricted to fertile women who manage to conceive and women who do not use oral contraceptives when getting pregnant or within 2 months before getting pregnant. To our knowledge only one previous epidemiological study has addressed the possible association between perfluorinated chemical exposure and menstrual disturbances. Though pointing toward different disturbances in cyclicity, both studies suggest that exposure to PFOA may affect the female reproductive function. This study contributes

to the limited knowledge on effects of exposure to PFOA and PFOS on female reproductive function and suggests that the female reproductive system may be affected by environmental exposure to PFOA.

STUDY FUNDING/COMPETING INTEREST(S): Supported by a scholarship from Aarhus University Research Foundation. The collection of questionnaire data and blood samples was part of the INUENDO project supported by The European Commission (Contract no. QLK4-CT-2001-00202), www.inuendo.dk. The Ukrainian part of the study was possible by a grant from INTAS (project 012 2205). Determination of PFOA and PFOS in serum was part of the CLEAR study (www.inuendo.dk/clear) supported by the European Commission's 7th Framework Program (FP7-ENV-2008-1-226217). No conflict of interest declared.

Key words: menstrual cycle / PFOS / PFOA

Introduction

Perfluorooctanate (PFOA) and perfluorooctane sulfonate (PFOS) are persistent man-made chemicals, with experimental evidence of reproductive toxicity (Lau *et al*., 2007). They belong to a class of perfluorinated chemicals (PFCs) used in consumer products, which are persistent in the environment and have been detected in wildlife and humans around the world (Giesy and Kannan, 2001; Kannan *et al*., 2004; Apelberg *et al*., 2007; Calafat *et al*., 2007; Fei *et al*., 2007). The mean half-life of PFOA and PFOS in human serum has been estimated as 3.4 and 5.4 years, respectively (Olsen *et al*., 2007). These relatively long half-lives in humans have raised concerns about potential health effects.

PFOA and PFOS may interfere with reproductive function, but evidence in humans is limited and inconsistent. Exposure to PFOA and PFOS at the levels found in the general Danish population may reduce fecundity, as indicated by irregular menstrual periods and increased time to pregnancy (TTP) as seen in a recent study (Fei *et al*., 2009). However, other studies found no association between levels of PFCs and TTP or subfecundity (Vestergaard *et al*., 2012; Whitworth *et al*., 2012).

Menstruation is the most frequent and easily observable event associated with female reproduction. It is the primary marker of ovarian function, and menstrual dysfunction is a major cause of infertility (Van Eijkeren *et al*., 1989; Harlow and Ephross, 1995). Hence, menstruation can be viewed as a proxy of female fecundity (Buck Louis *et al*., 2011). Most women experience numerous changes in their menstrual cycle patterns during their reproductive life (Harlow and Ephross, 1995) with little change in length and variability between the ages of 19 and 49 and a tendency for longer cycles and high cycle variability in the years immediately following menarche and preceding menopause (Treloar *et al*., 1967; Belsey and Pinol, 1997). Long cycles and cycle irregularities have been associated with lower fecundity (Jensen *et al*., 1999; Kolstad *et al*., 1999; Small *et al*., 2010; McLain *et al*., 2012; Mumford *et al*., 2012) and menstrual cycle disturbances and variability have been associated with several factors, including age (Collett *et al*., 1954; Harlow *et al*., 2000), BMI (Jensen *et al*., 1999), smoking (Rowland *et al*., 2002), extreme exercise (Chen and Brzyski, 1999), work stress (Hatch *et al*., 1999), organic solvents (Cho *et al*., 2001) and other chemical compounds such as polychlorinated biphenyls (PCBs) (Buck Louis *et al*., 2011). *In vitro* studies with human cells have indicated that PFOS and PFOA have both estrogenic and anti-estrogenic effects (Henry and Fair, 2013) and that PFOS is capable of altering steroidogenesis (Kraugerud *et al*., 2011). In humans, a recent study with Greenlandic women found that several PFCs, including PFOS and PFOA, have estrogenic effects (Bonefeld-Jorgensen *et al*., 2011) while another study found that only PFOS was inversely associated with estradiol levels (Knox *et al*., 2011). Thus, the chemicals may affect the complex hormonal system involved with reproduction, yet the magnitude and direction seems unclear. Estradiol and progesterone are essential hormones controlling the menstrual cycle and, therefore, it is possible that chemicals disrupting their production/function may disturb menstrual cyclicity.

The objective of this study was to investigate the association between measured PFOS and PFOA exposure and menstrual cycle length and irregularities in women from Greenland, Poland and Ukraine who managed to achieve pregnancy.

Materials and Methods

Study population

The study population consisted of pregnant women from the INUENDO cohort. The women were enrolled during antenatal care visits between June 2002 and May 2004 at local hospitals in 19 municipalities and settlements in Greenland, at a large central hospital and a collaborating hospital in Warsaw, Poland and at three hospitals and eight antenatal clinics in Kharkiv, Ukraine. With few exceptions, the antenatal care program covered all pregnant women in these localities.

All together 3833 pregnant women from these three localities were informed about the study and encouraged to participate. All women were asked to fill in a questionnaire at a face-to-face interview with a midwife or an obstetrician and have a blood sample drawn. A more detailed description of the recruitment procedure and participation can be found elsewhere (Toft *et al*., 2005). A questionnaire was developed in English and translated to the language of the respective study population. A general criterion for eligibility was that the women should be at least 18 years of age and born in the country where the study was conducted. A total of 1735 (45.3%) women were interviewed and the participation rates were 91.3% in Greenland, 69.1% in Warsaw, Poland and 26.3% in Kharkiv, Ukraine. Altogether, 1623 women were included in our main analysis.

Non-participating and excluded women

Reasons for not participating varied across regions. In Greenland, 32 women were living in inaccessible areas and 35 refused to participate. In Ukraine, one of the reasons for not participating was concern that collection of a blood sample would imply a risk for the pregnancy and the baby but other reasons were not systematically registered. No information on causes of non-participation was available from women from Poland. A total of 1735 women were interviewed. However, 99 women were excluded because they used oral contraceptives when getting pregnant or within 2 months before getting pregnant, leading to control of their menstrual cycle. Another 13 were excluded due to average cycle length <16 days, which was interpreted as a reporting error.

Exposure to PFOA and PFOS

Blood samples were drawn from a cubital vein into 10 ml vacuum tubes for serum collection without additives (Becton Dickinson, Maylan, France). After cooling to room temperature, the tubes were centrifuged at 4000*g* for 15 min. Serum was transferred with ethanol-rinsed Pasteur pipettes to ethanol-rinsed brown glass bottles (Termometerfabriken, Gothenburgh, Sweden). Sera were stored at −20°C until shipment. After arrival at the analyzing laboratory at the Department of Occupational and Environmental Medicine in Lund, Sweden, the samples were kept at −80°C until analyzed. The analysis of PFOS and PFOA was performed by liquid chromatography (LC) tandem mass spectrometry. Aliquots of 100 μl serum were added with labeled internal standards for both compounds and the proteins were precipitated with acetonitrile and vigorously shaking for 30 min. The samples were thereafter centrifuged and the supernatant analyzed using a LC (UFLC^{XR}, SHIMADZU Corporation, Kyoto, Japan) connected to a hybrid triple quadrupole linear ion trap mass spectrometer (QTRAP 5500, AB Sciex, Foster City, CA, USA). A more detailed description can be found elsewhere (Lindh *et al*., 2012). All values were above limits of detection (0.2 and 0.04 ng/ml) for PFOS and PFOA, respectively) and the coefficient of variation (CV) of duplicate samples worked-up and analyzed on different days was 9% and 11% for PFOS and PFOA, respectively. The analyses of PFOS and PFOA are part of the Erlangen Round Robin inter-laboratory control program with results within the tolerance limits. The laboratory was blinded to any information on the pregnant women.

Outcome

The participants were asked about their menstrual cycle characteristics in the period before getting pregnant (i.e. when the couple started having sexual intercourse without using any birth control to prevent pregnancy) using a validated questionnaire (Juul *et al*., 1999). Specifying that the women should think about the time period before getting pregnant, the following question was used to assess menstrual cycle characteristics among the women: 'How long was it from the start of one menstrual bleeding to the start of the next bleeding?'. The participants could reply either a number of days, give the range of days or reply that they had no bleeding at all or could not remember or did not know. Thus, each woman contributed one average cycle length, either as an exact number of days or a range of days which was subsequently calculated into an average cycle length. A number of women ($n = 155$) did not remember or did not know their cycle length. Women who reported a difference of 7 days or more in cycle lengths between months were defined as having irregular cycles (Kolstad *et al*., 1999; Small *et al*., 2007). Short cycles were defined as 24 days or less, whereas long cycles were 32 days or more based on the distribution of cycle length and fertility in relation to cycle length in prospective studies (Kolstad *et al*., 1999; Small *et al*., 2007). Information on potential confounders (age, BMI, parity, smoking, education and drinking) was obtained with reference to the same time as information regarding menstrual cycle length. Furthermore, the women were asked about their age at first menstrual period.

Ethical approval

The study was approved by the local ethical committees and written informed consent was obtained from all included women.

Statistical analysis

Missing information

The original dataset contained a relatively large amount of missing data. In total, exposure-data were missing for 275 (16.9%) of the 1623 participants, and outcome-data were missing for 364 (22.4%) of the included women. The number of missing values for the covariates ranged from 33 (2.0%) to 177 (10.9%) in pooled data. Complete-case analysis may lead to biased estimates as women with complete data on all covariates may represent a biased sample of the population of interest (Sterne *et al*., 2009). We addressed the missing data problem by using multiple imputation to complete the dataset, thus allowing us to include persons with incomplete data in the main analysis as multiple imputation produce unbiased, more precise and powerful estimates, assuming that data are missing at random (i.e. the probability that data are missing does not depend on unobserved data and thus is correlated with other variables included in the analysis) (Rubin and Little, 1987). Briefly, multiple imputation makes use of known subject characteristics from the original dataset. Multiple different imputed datasets ($m > 1$) are created, as each missing value is replaced with a set of random plausible values. The values are generated from known covariates and distributions (Mumford *et al*., 2011), insuring an appropriate variability. Subsequently, the *m* complete datasets are analyzed and the results are combined by the rules of Rubin (Rubin and Little, 1987; Sterne *et al*., 2009).

We generated 100 complete datasets with imputed data. The following variables were included as predictors in the main imputation model: PFOS, PFOA, mean length of cycle, minimum length of cycle, maximum length of cycle, irregular cycle, age at menarche, age at pregnancy, pre-pregnancy BMI, smoking, parity and educational level. We made sensitivity analyses by using different imputation models, and by creating a varying number of datasets (20 and 200). In addition, we compared results when using the strategy of complete-case analysis (i.e. when only subjects with full information on the variables needed in any given analysis were included) with analysis based on the imputed dataset. All imputations were implemented with the ice add-on command, and the built-in mi estimate command of STATA 12 (Stata Corporation, College Station, TX, USA).

Data analysis

The association between PFOS and PFOA exposure and menstrual cycle length and regularity was modeled by multiple logistic regression analysis. Data were stratified by country into three groups (Greenland, Poland and Ukraine), and PFOS and PFOA exposure were each categorized into tertiles with the lowest tertile as reference level. As exposure levels varied between countries, the levels of low, middle and high exposure rates are not comparable across countries. We checked for effect modification between each of the continuous exposure variables and country. No significant effect modification was identified and consequently we made pooled estimates of all three countries adjusting for country. Odds ratios (ORs) for short cycles and long cycles were estimated by logistic regression in separate models, using only normal cycles defined as cycle length between 25 and 31 days as reference. All outcome variable estimates are presented as adjusted ORs with 95% confidence intervals (CIs). The following potential confounders were identified *a priori*: age at menarche (continuous), age at pregnancy (continuous), parity (continuous), prepregnancy BMI (continuous) and smoking habits (smoker/non-smoker; Collett *et al*., 1954; Hornsby *et al*., 1998; Jensen *et al*., 1999; Harlow *et al*., 2000; Rowland *et al*., 2002; McLain *et al*., 2012). Adjusting for both age at pregnancy and age at menarche are equivalent to adjusting for years since menarche and as we find it more natural we have chosen the former way of presenting the variables. We did a stringent prioritization of the potential confounders for each outcome and included the strongest confounders in the regression models based on an *a priori* identification and literature search. In addition, logistic regression analysis with continuous PFOS and PFOA as covariates (log transformed) was performed in a continuous analysis, where a high OR indicates higher odds of cycle disturbances due to higher exposure levels. In a subanalysis we included adjustment for gestational age (GA) at the time of blood sampling. As previous studies have suggested that parity might be an effect modifier, we conducted a subanalysis stratified by parity (nulliparous/parous). Other subanalyses were performed without the adjustment for parity, as parity is likely to be a consequence of menstrual disturbances. Also, a subanalysis was done to account for the possible relation between long or irregular cycles and prolonged

TTP (>12 months). *A priori*, we had no prospects of whether a possible effect of exposure would increase the odds of short or long cycles. In a subanalysis, we made a linear regression analysis between continuous PFOS and PFOA (log transformed) and continuous mean cycle length. Statistical analyses were performed using the STATA software version 12.1 (Stata Corporation, College Station, TX, USA). The term 'statistically significant' is used to denote a *P*-value <0.05.

Results

Characteristics of the 1623 women included in the study are presented in Table I. Short cycles (≤24 days) were primarily found among women from Ukraine (10.2%) whereas only a small number of short cycles were encountered among women from Greenland (1.8%) and Poland (1.6%). The highest prevalence of long cycles (≥32 days) was found among women from Poland (19.6%), but only among 7.9% of the women from Ukraine. Age at pregnancy differed between study populations with the lowest median age among women from Ukraine and the highest among women from Poland. Furthermore, the covariates GA, parity, BMI and particularly smoking varied between countries.

The median serum concentrations of PFCs differed considerably between countries, with the following PFOS and PFOA concentrations, respectively; Greenland: 20.2 and 1.8 ng/ml, Poland: 8.0 and 2.7 ng/ml and Ukraine: 5.0 and 1.0 ng/ml (Table I). The differences in exposure profiles are further presented in Table II describing the exposure tertiles stratified by country. The highest exposure level of PFOS was found among women from Greenland while much lower exposure levels were found among women from Poland and Ukraine. In contrast, exposure levels of PFOA were more similar across countries with the highest exposure detected among the Polish women and lower levels among women from Greenland and Ukraine.

As the association between continuous exposure levels and menstrual cycle characteristics were consistent across countries, we were able to make a joined estimate of the effects. Adjusted ORs with 95% CI for irregular, short and long cycles in the women according to exposure levels of PFOS and PFOA are given in Table III. The results indicated a

Table I Characteristics of the study population and presentation of missing data (*N* = 1623).

	Greenland (*n* = 528)	Poland (*n* = 452)	Ukraine (*n* = 643)	Pooled (*N* = 1623)
Exposure				
PFOS (ng/ml), median (p10; 90)[a]	20.2 (12.0; 36.9)	8.0 (5.2; 12.1)	5.0 (2.9; 8.0)	8.0 (3.6; 25.6)
PFOA (ng/ml), median (p10; 90)	1.8 (1.0; 3.0)	2.7 (1.5; 4.3)	1.0 (0.5; 1.7)	1.5.0 (0.7; 3.1)
Missing information on exposure, *n* (%)	26.0 (4.9)	207.0 (45.8)	42.0 (6.5)	275.0 (16.9)
Outcome				
Cycle length (days), median (p10; 90)	28.0 (28.0; 31.5.0)	29.0 (27.0; 34.0)	28.0 (24.0; 31.0)	28.5 (26.0; 32.0)
Irregular cyclus[b], *n* (%)	91.0 (18.6)	30.0 (8.0)	20.0 (5.1)	141.0 (8.7)
Short cycle (≤24 days), *n* (%)	9.0 (1.8)	6.0 (1.6)	40.0 (10.2)	55.0 (3.4)
Long cycle (≥32 days), *n* (%)	45.0 (9.2)	74.0 (19.6)	31.0 (7.9)	150.0 (9.2)
Missing information on outcome, *n* (%)	39.0 (7.4)	75.0 (16.6)	250.0 (38.9)	364.0 (22.4)
Covariates				
Age at menarche (years), median (p10; 90)	13.0 (11.0; 14.0)	13.0 (12.0; 15.0)	13.0 (11.0; 14.0)	13.0 (11.0; 15.0)
Missing, *n* (%)	17.0 (3.2)	33.0 (7.3)	31.0 (4.8)	81.0 (5.0)
Age at pregnancy (years), median (p10; 90)	25.8 (19.5; 36.6)	28.7 (25.4; 33.7)	24.4 (19.5; 32.1)	26.6 (20.1; 34.2)
Missing, *n* (%)	12.0 (2.3)	14.0 (3.1)	37.0 (5.8)	63.0 (3.9)
GA[c], median (p10; 90)	24.0 (11.9; 36.4)	33.7 (28.7; 37.9)	22.5 (9.0; 40.3)	28.8 (11.3; 38.6)
Missing, *n* (%)	40.0 (7.6)	36.0 (8.0)	61.0 (9.5)	137.0 (8.4)
Parity, *n* (%)				
Nulliparous	151.0 (28.6)	407.0 (90.0)	497.0 (77.3)	1055.0 (65.0)
Parous	367.0 (69.5)	36.0 (8.0)	128.0 (19.9)	531.0 (32.7)
Missing, *n* (%)	10.0 (1.9)	9.0 (2.0)	18.0 (2.8)	37.0 (2.3)
Prepregnancy BMI, median (p10; 90)	23.8 (19.8; 30.1)	20.8 (18.2; 24.8)	21.2 (18.2; 25.7)	21.8 (18.6; 27.3)
Missing, *n* (%)	11.0 (2.1)	6.0 (1.3)	16.0 (2.5)	33.0 (2.0)
Smoking, *n* (%)				
Yes	456.0 (88.2)	143.0 (31.6)	193.0 (30.0)	654 (40.3)
No	61.0 (11.8)	264.0 (58.4)	329.0 (51.2)	792 (48.8)
Missing, *n* (%)	11.0 (2.1)	45.0 (10.0)	121.0 (18.8)	177 (10.9)

PFOS, perfluorooctane sulfonate; PFOA, perfluorooctanoate.
[a](p10;90); 10;90% percentiles.
[b]Irregular cycle is defined as ≥7 days of variation.
[c]GA when the blood sample was drawn.

Table II Description of the exposure tertiles, stratified by country and pooled data.

	Greenland	Poland	Ukraine	Pooled
PFOS				
Low	4.1–16.9 ($n = 168$)	1.6–7.1 ($n = 82$)	0.7–4.2 ($n = 201$)	0.8–5.9 ($n = 450$)
Middle	17.0–23.9 ($n = 167$)	7.2–9.5 ($n = 82$)	4.3–5.8 ($n = 200$)	6.0–13.3 ($n = 449$)
High	24.0–87.3 ($n = 167$)	9.6–21.3 ($n = 81$)	5.9–18.1 ($n = 200$)	13.4–87.3 ($n = 449$)
PFOA				
Low	0.5–1.5 ($n = 168$)	0.5–2.2 ($n = 82$)	0.2–0.8 ($n = 201$)	0.2–1.1 ($n = 450$)
Middle	1.6–2.2 ($n = 167$)	2.3–3.1 ($n = 82$)	0.9–1.1 ($n = 200$)	1.2–1.9 ($n = 449$)
High	2.3–5.1 ($n = 167$)	3.2–9.8 ($n = 81$)	1.2–9.8 ($n = 200$)	2.0–9.8 ($n = 449$)

PFOS and PFOA are measured in ng/ml.
PFOS, perflourooctane sulfonate; PFOA, perfluorooctanoate.

Table III OR (95% CI) for irregular, short or long cycles, pooled estimates of all three countries: logistic regression analysis—multiple imputation.

	Irregular cycle (adjusted OR)	Short cycle (adjusted OR)	Long cycle (adjusted OR)
PFOS			
Low	1.0 (ref.)	1.0 (ref.)	1.0 (ref.)
Middle	1.1 (0.6; 2.1)	0.8 (0.4; 1.6)	1.3 (0.8; 2.2)
High	1.7 (0.8; 3.5)	1.3 (0.5; 3.9)	1.2 (0.6; 2.5)
Continuous[a]	1.2 (0.9; 1.8)	1.0 (0.6; 1.7)	1.1 (0.8; 1.6)
PFOA			
Low	1.0 (ref.)	1.0 (ref.)	1.0 (ref.)
Middle	1.3 (0.8; 2.3)	0.7 (0.4; 1.5)	1.4 (0.8; 2.3)
High	1.3 (0.7; 2.3)	0.7 (0.3; 1.8)	1.8 (1.0; 3.3)
Continuous[b]	1.3 (0.8; 1.9)	0.8 (0.5; 1.4)	1.5 (1.0; 2.1)

Irregular cycle is defined as $\geq$ 7 days of variation. Short cycles and long cycles are compared with normal cycles as reference (25– 31 days). Adjusted for age at menarche (continuous), age at pregnancy (continuous), parity (continuous), prepregnancy BMI (continuous), smoking (smoker/ non-smoker) and country (categorical).
PFOS, perflourooctane sulfonate; PFOA, perfluorooctanoate.
[a]Log-transformed PFOS as a continuous variable.
[b]Log-transformed PFOA as a continuous variable.

statistically significant association between PFOA exposure and long cycles with OR 1.8 (95% CI: 1.0; 3.3) when comparing the highest tertile of exposure with the lowest. Furthermore, the continuous analysis showed that for each log-unit increase in PFOA exposure the odds of experiencing long cycles increased by a factor 1.5 (95% CI: 1.0;2.1). No statistically significant associations were observed between PFOS exposure and menstrual cycle characteristics. However, a tendency toward irregular cycle with increasing PFOS was observed when comparing the highest tertile of exposure with the lowest [OR 1.7 (95% CI: 0.8; 3.5)]. The Supplementary data, Table SI presents crude and adjusted ORs when stratifying by country. Owing to the sparse number of participants with short cycles among women from Greenland and Poland, no statistical analysis on this outcome could be performed.

Table IV shows adjusted ORs from the complete-case analysis. Furthermore, it presents the number of persons in each exposure tertile as well as the number of cycle characteristics encountered in each of these. Results based on multiple imputation of missing values (Table III) were similar to those based on complete-case analysis (Table IV). The Supplementary data, Table SII presents crude and adjusted ORs from the complete-case analysis when stratifying by country. Adjusting for GA at the time of blood sampling did not change the results essentially (data not shown). A subanalysis with stratification by parity (nulliparous/parous) is presented in the Supplementary data, Tables SIII and SIV. Restricting the analysis to only nulliparous women did not alter the observed association between PFOA exposure and long cycles with OR 1.8 (95% CI: 0.9; 3.9) when comparing the highest tertile of exposure with the lowest. As the number of included women in each stratum by definition is smaller than the total the CIs within strata are wider than in the joint analysis, which made the strata-specific association's only borderline significant. Subanalyses without adjustment for parity showed comparable results with OR 2.0 (95% CI: 1.2; 3.3) for the association between PFOA exposure and long cycles and OR 1.7 (95% CI: 0.8; 3.8) for irregular cycle with increasing PFOS. Crude analyses between cycle characteristics and prolonged TTP (>12 months) showed a significant association between irregular cycles and prolonged TTP [OR 1.7 (95% CI: 1.1; 2.5)] while the association between long cycles and prolonged TTP was only borderline significant [OR 1.3 (95% CI: 0.9; 1.9)].

A linear regression analysis between exposure and the average cycle length is presented in Table V. The analysis showed a significant association between exposure to PFOA and difference in the average cycle length when comparing the highest tertile of exposure with the lowest. No significant association was observed when exposed to PFOS.

Discussion

We observed longer menstrual cycles with higher exposure to PFOA. Furthermore, tendencies toward increased odds of irregular cycles with increased exposure to both PFOS and PFOA were observed, although not statistically significant. To the best of our knowledge, this is the largest study to examine a possible relation between PFOS and PFOA exposure and both menstrual cycle length and regularity in humans.

Data concerning reproductive toxicity of PFCs are limited and inconsistent (Whitworth *et al.*, 2012). Several animal studies have reported that exposures to PFOS and PFOA affects sex hormone homeostasis, increases the incidence of pregnancy loss and decreases the number of regular estrous cycles (Case *et al.*, 2001; Austin *et al.*, 2003; Lau *et al.*, 2007; Wolf *et al.*, 2007), while others found no alteration in fertility parameters, including estrous cycling after exposure to PFOS (Luebker *et al.*, 2005) or PFOA (Butenhoff *et al.*, 2004). However, the generalizability of results from animal studies to human populations remains uncertain since the exposure profile is much higher in these studies compared with the background of human exposure (Butenhoff *et al.*, 2004). In continuation, the toxicokinetics of different PFCs differ considerably

Table IV OR (95% CI) for irregular, short or long cycles, pooled estimates of all three countries: logistic regression analysis—complete-case analysis.

	Irregular cycle			Short cycle			Long cycle		
	n	Number of cycle characteristics	Adjusted OR	*n*	Number of cycle characteristics	Adjusted OR	*n*	Number of cycle characteristics	Adjusted OR
PFOS									
Low	219	11	1.0 (ref.)	199	17	1.0 (ref.)	201	19	1.0 (ref.)
Middle	292	28	1.1 (0.5; 2.7)	242	8	0.6 (0.2; 1.5)	281	43	1.2 (0.6; 2.3)
High	393	76	1.3 (0.4; 3.7)	330	9	2.4 (0.5; 14.7)	382	37	0.6 (0.2; 1.4)
Continuous[a]	904	115	1.0 (0.6; 1.6)	771	34	0.7 (0.3; 1.5)	864	99	0.7 (0.4; 1.2)
PFOA									
Low	233	15	1.0 (ref.)	215	17	1.0 (ref.)	214	16	1.0 (ref.)
Middle	315	44	1.5 (0.8; 2.8)	268	8	0.6 (0.2; 1.4)	305	30	1.4 (0.7; 2.7)
High	356	56	1.6 (0.8; 3.1)	288	9	0.7 (0.2; 1.9)	345	53	2.1 (1.1; 4.3)
Continuous[b]	904	115	1.4 (0.9; 2.2)	771	34	0.7 (0.3; 1.5)	864	99	1.7 (1.1; 2.6)

Irregular cycle is defined as ≥7 days of variation. Short cycles and long cycles are compared with normal cycles as reference (25–31 days). Adjusted for age at menarche (continuous), age at pregnancy (continuous), parity (continuous), prepregnancy BMI (continuous), smoking (smoker/non-smoker) and country (categorical).
PFOS, perflourooctane sulfonate; PFOA, perfluorooctanoate.
[a]Log-transformed PFOS as a continuous variable.
[b]Log-transformed PFOA as a continuous variable.

Table V Chemical exposure and average difference in cycle length (days): linear regression analysis ($N = 1623$).

Exposure	Average difference in cycle length [Regression coefficient (95% CI)]
PFOS	
Low	(ref.)
Middle	0.37 (−0.34;1.08)
High	0.24 (−0.74;1.23)
Continuous[a]	0.08 (−0.42;0.58)
PFOA	
Low	(ref.)
Middle	0.43 (−0.17;1.04)
High	0.91 (0.22;1.60)
Continuous[b]	0.55 (0.05;1.04)

Adjusted for age at menarche (continuous), age at pregnancy (continuous), parity (continuous), prepregnancy BMI (continuous), smoking (smoker/non-smoker) and country (categorical).
PFOS, perflourooctane sulfonate; PFOA, perfluorooctanoate.
[a]Log-transformed PFOS as a continuous variable.
[b]Log-transformed PFOA as a continuous variable.

between animal species (Lau *et al.*, 2007), half-lives are very different in humans compared with animals (Lau, 2012) and the estrous cycle in rodents may not be applied to humans.

Female exposure to PFOS and PFOA has in an epidemiological study been associated with irregular menstrual periods and increased TTP (Fei *et al.*, 2009). In contrast, another Danish study found no association between levels of PFCs and TTP among first-pregnancy planners (Vestergaard *et al.*, 2012) and a Norwegian study found no association between levels of PFCs and subfecundity when not stratifying by parity (Whitworth *et al.*, 2012). The latter two studies proposed reverse causality as an explanation of the prior findings when not stratifying by parity. However, the trend of reduced fecundability remained in the first mentioned Danish study (Fei *et al.*, 2009) when stratifying by parity and thus the authors found limited evidence for reverse causation as an explanation for their results (Fei *et al.*, 2012). Our findings are in line with this as restriction to only nulliparous women in the stratified subanalysis did not alter the observed association between PFOA and long cycle and thus this study does not support previous observations that associations between PFCs and reproductive function are only found in parous women. As discussed in the paper by Fei *et al.* (2012), parity is a predictor of PFC levels in blood and also a function of fecundity and therefore we adjusted for parity in the multivariate analyses. Parity might be an intermediary variable, but even after adjusting for parity the association remained statistically significant. The biological mechanisms by which exposure to PFCs may alter female fecundity are speculative. Possible mechanisms include direct or indirect action on the hypothalamic–pituitary–gonadal axis (Austin *et al.*, 2003), but like most toxicants PFCs are likely to have multiple modes of action.

To our knowledge, the only previous epidemiological study touching upon a possible association between exposure to PFOS and PFOA and menstrual cycle characteristics was performed by Fei *et al.* (2009). The study was performed among 1240 women from the Danish National Birth Cohort and found that exposure to both PFOS and PFOA was associated with irregular menstrual periods when comparing upper three quartiles of exposure with the lowest (reference). Their sample collection was carried out in 1996–2002, while the samples in our study were collected in 2002–2004. It is well recognized that serum levels of PFOS and PFOA have declined since the year of 2000. In the general US population, the decline has been ~30% from year 2000 to 2003–2004 (Calafat *et al.*, 2007). This decline might partly explain the great difference in mean concentrations of PFOS and PFOA reported by Fei (35.3

and 5.6 ng/ml, respectively) and the medians presented in our study (Greenland: 20.2 and 1.8 ng/ml, Poland: 8.0 and 2.7 ng/ml and Ukraine: 5.0 and 1.0 ng/ml, respectively), though differences in exposure profiles between countries are believed to be a major cause. Also, Fei *et al.* (2009) measured exposure levels early in the pregnancy, whereas our measurements in general were performed later in the pregnancy. In another study, Fei *et al.* showed a decline in levels of PFOS and PFOA during pregnancy, which may be an additional reason for the observed difference in exposure levels (Fei *et al.*, 2007); however, adjusting for GA at the time of blood sampling did not change our estimates (data not shown). Even though pointing toward different findings, both Fei's and our results support that the menstrual cycle may be vulnerable to PFOA exposure.

There are some limitations in our study. First, participation rates varied considerably between countries with rates of 91.3% in Greenland and only 26.3% in Ukraine. Those participating may not be representative of the target population, but since the women were unaware of their exposure levels this is unlikely to have influenced their choice of participation. Furthermore, it is important to state that our study population was restricted to women who managed to conceive and avoid early pregnancy loss. As exposure to PFCs potentially affects menstrual cyclicity to the point of impacting fecundity, women with highly affected cyclicity are underrepresented in our study, leading to a selected study group that overrepresents the most fertile part of the general population. The exclusion of women using oral contraceptives further limits the generalizability of our results to women who do not use oral contraceptives 2 months or less before or when getting pregnant. We cannot rule out the possibility of some women having their cycles artificially altered toward regularity by previous use of oral contraceptives. However, a possible misclassification is unlikely to be related to the exposure levels and thus is believed to be non-differential.

Information on menstrual cycle characteristics was obtained through retrospective information regarding the period when trying to become pregnant. Records of menstrual cycles offer a non-invasive, immediate measure of a women's reproductive health, but the accuracy of retrospective reports on menstrual data are not perfect and may arguably lead to some degree of misclassification. Hence, several studies have examined the use of retrospective reports of the menstrual cycle compared with prospective information. Recent studies observed a poor to moderate agreement between self-reported menstrual cycle length and prospectively observed cycle length (Small *et al.*, 2007; Jukic *et al.*, 2008). Jukic *et al.* (2008) found that women on average overestimated the cycle length by 0.7 days, although reporting by sexually active women was more accurate. Although pointing toward considerable measurement errors in self-reported cycle length, Small *et al.* (2007, 2010) stated that including both measures on length and variability in the analysis of menstrual cycles reduce the amount of measurement errors. In contrast, other studies have shown good correlations between self-report and the average, actual cycle length (Steiner *et al.*, 2001; Creinin *et al.*, 2004) although a narrow selection criteria (including only cycle length of 21–35 days) limits their generalizability. However, in individual cases there might be a risk of recall errors, but differential misclassification of outcomes is unlikely in our study since none of the women were aware of their PFC levels. Furthermore, it is possible that self-report by the women in our study is more accurate, since most of them likely tried to get pregnant, whereas this was the case for only 22% of the women in the study by Small *et al.* (2007). Study results could be strengthened by assessing outcome information prospectively through menstrual diaries. Yet, this approach has obvious disadvantages with respect to cost and compliance compared with questionnaires filled out retrospectively.

We determined cut-points for all three outcome variables based on existing literature (Kolstad *et al.*, 1999; Small *et al.*, 2007); however, several other definitions regarding short, long and irregular cycles have been proposed (Jukic *et al.*, 2008; Fruscalzo *et al.*, 2010; Yang *et al.*, 2011). We did not have substantial information on some potentially important determinants regarding menstrual cycle characteristics, including stress, physical activity, chronic diseases and gynecological disorders, and thus cannot exclude the possibility of unmeasured confounding by these factors. However, the missing variables are believed to be determinants of the menstrual cycle but not considered to be associated with the exposure and are therefore most likely not confounders for the association between the exposure and menstrual cycle. Furthermore, we cannot rule out the possibility of residual confounding due to somewhat broad categories of the included variables.

The strengths of our study include the specificity of the exposure classification, relying on bio-monitored data measured directly to assess the women's individual exposures to PFOS and PFOA. Even though exposure was not measured at the same time as the recalled menstrual cycle characteristics the chemical stability possessed by PFCs (Fromel and Knepper, 2010) and the long half-lives of PFOS and PFOA in humans make the biological measurement an important methodological strength, which minimizes exposure misclassification.

A further strength is the use of identical methods for assessing information on exposure, outcome and confounders in all three populations. Comparison of health outcomes in regions with highly contrasting exposure levels may provide clues regarding environmental causes of disease, which are otherwise impossible to detect within populations (Rose, 1985; Jonsson *et al.*, 2005). If PFOS and PFOA have substantial effects on menstrual cycle characteristics we would expect it to be observed in all three populations, or at least in the population with the highest level of exposure, and we indeed observed associations pointing in the same direction in the three countries.

Our findings suggest that the female reproductive system may be sensitive to background exposure to PFOA. A possible effect on the reproductive system is in line with a recent study, where higher concentrations of LH and FSH in human male offspring due to PFOA exposure were found (Vested *et al.*, 2013). LH and FSH are essential hormones controlling the menstrual cycle, and hence, disturbances in these hormones may disturb menstrual cyclicity. The link between menstrual cycle disturbances and longer TTP was confirmed in a subanalysis of our data. As longer cycle and higher cycle variability is associated with lower fecundity our findings indicate that particularly PFOA exposure might have clinical implications for women desiring to conceive. Standardized definitions of cycle characteristics are needed in the future for researchers to accurately compare results, thus enabling them to draw valuable conclusions.

In conclusion, our findings on 1623 pregnant women from the INUENDO cohort in Greenland, Poland and Ukraine suggest longer menstrual cycles with higher exposure to PFOA. Exposure to PFOS is unlikely to be a main cause of menstrual disturbances in humans.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

J.L., C.H.R.H., B.B.H., H.S., J.P.B. and G.T.: contributions to conception, design, data interpretation. J.L., C.H.R.H., B.B.H., H.S., J.P.B., B.A.G.J., C.H.L., V.Z., H.S.P., J.K.L. and G.T.: paper revision and approval of the submitted version. B.A.G.J., C.H.L., V.Z., H.S.P., J.K.L.: acquisition of data. J.L., C.H.R.H., B.B.H., H.S. and G.T.: data analysis. J.L.: drafting the paper.

Funding

J.L. scholarship was granted by the Aarhus University Research Foundation. The collection of questionnaire data and blood samples was part of the INUENDO project supported by The European Commission (Contract no. QLK4-CT-2001-00 202), www.inuendo.dk. The Ukrainian part of the study was possible by a grant from INTAS (project 012 2205). Determination of PFOA and PFOS in serum was part of the CLEAR study (www.inuendo.dk/clear) supported by the European Commission's 7th Framework Program (FP7-ENV-2008-1-226217).

Conflict of interest

No conflict of interest declared.

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