Human Reproduction, Vol.27, No.3 pp. 873–880, 2012
Advanced Access publication on January 13, 2012 doi:10.1093/humrep/der450

human reproduction

ORIGINAL ARTICLE *Reproductive endocrinology*

Association between perfluorinated compounds and time to pregnancy in a prospective cohort of Danish couples attempting to conceive

Sonja Vestergaard[1,*], Flemming Nielsen[1], Anne-Maria Andersson[2], Niels Henrik Hjøllund[3,4], Philippe Grandjean[1,5], Helle Raun Andersen[1], and Tina Kold Jensen[1,2]

[1]Department of Environmental Medicine, Institute of Public Health, University of Southern Denmark, J.B. Winsloews Vej 17A, DK-5000 Odense C, Denmark [2]Department of Growth and Reproduction, The National Hospital, DK-2100 Copenhagen, Denmark [3]Department of Occupational Medicine, Herning Regional Hospital, DK-7400 Herning, Denmark [4]Department of Clinical Epidemiology, Aarhus University Hospital, DK-8200 Aarhus N, Denmark [5]Department of Environmental Health, Harvard School of Public Health, Boston, MA 02115-6018, USA

*Correspondence address. Tel: +45-6550-3768; Fax: +45-6591-1458; E-mail: svestergaard@health.sdu.dk

Submitted on August 17, 2011; resubmitted on November 24, 2011; accepted on December 7, 2011

BACKGROUND: Perfluorinated chemicals (PFCs) have been widely used and have emerged as important food contaminants. A recent study on pregnant women suggested that PFC exposure was associated with a longer time to pregnancy (TTP). We examined the association between serum concentrations of PFCs in females and TTP in 222 Danish first-time pregnancy planners during the years 1992–1995.

METHODS: The couples were enrolled in the study when discontinuing birth control and followed for six menstrual cycles or until a clinically recognized pregnancy occurred. Fecundability ratio (FR) was calculated using discrete-time survival models. In addition, odds ratio (OR) for TTP >6 cycles was calculated.

RESULTS: OR for TTP >6 cycles for those with PFC concentrations above the median were 0.96 [95% confidence interval (CI): 0.54–1.64] for perfluorooctane sulfonic acid (PFOS), the major PFC, compared with those below the median. FRs for those with PFOS concentrations above the median were 1.05 (95% CI: 0.74–1.48) compared with those below the median. Other PFCs showed the same lack of association with TTP. The results were not affected by adjustment for covariates. PFOS and perfluorooctanoic acid concentrations were similar to those observed in a previous Danish study.

CONCLUSIONS: These findings suggest that exposure to PFCs affects TTP only to a small extent, if at all.

Key words: perfluorinated compounds / time to pregnancy / fecundability / discrete-time analysis / cohort

Introduction

Perfluorinated compounds (PFCs) are worldwide contaminants of food and drinking water (EFSA, 2008; Halldorsson *et al.*, 2008; Trudel *et al.*, 2008; Haug *et al.*, 2011; Trier *et al.*, 2011; Xiao *et al.*, 2012). Experimental evidence exists with regard to reproductive toxicity for the two main PFCs, perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA; EFSA, 2008). Despite the extensive toxicological data, adverse effects in humans, if any, are inadequately characterized (Steenland *et al.*, 2010), and several PFCs may be of concern in this regard (Fei and Olsen, 2010). Reproductive toxicity in humans has been suggested by decreased fetal growth (Fei *et al.*, 2008; Olsen *et al.*, 2009), and a Danish study investigated the possible association with time to pregnancy (TTP; Fei *et al.*, 2009). TTP is a sensitive marker of fecundability (Rachootin and Olsen, 1982) and among 1240 pregnant women, the subjects with serum PFOS in the highest quartile had a 26% [fecundability ratio (FR) of 0.74; 95% confidence interval (CI): 0.58, 0.93] reduced chance of becoming pregnant within the same cycle as compared with women in the lowest quartile (Fei *et al.*, 2009). However, this study included only those women who had already attained pregnancy, thus creating possible selection bias along with information bias, as recognized by the authors. Furthermore, their data were susceptible to bias related to reverse causality (Whitworth *et al.*, 2011). To explore the possible

reproductive risk, we chose to examine PFC exposures in a prospective study of women attempting pregnancy for the first time, a design not liable to bias from reverse causality.

Methods

Sample description

From 1992 to 1995 a total of 430 couples were recruited after a nationwide mailing of personal letters to 52 255 trade union members (metal workers, office workers, nurses, and day-care workers) who were of 20–35 years old, lived with a partner and had no children. Couples without previous reproductive experience who intended to discontinue contraception to become pregnant were eligible for enrolment. The exact number of eligible couples in the source population of 52 255 people was unknown. Under the assumption that 75% of pregnancies in Denmark are planned, an average participation rate of 16% was estimated by using data from union, age, parity and calendar-specific birth rates obtained from the Danish civil registration system. A detailed description of the study is provided elsewhere (Bonde *et al.*, 1998; Jensen *et al.*, 1998). The couples were enrolled into the study when they discontinued birth control and were followed for six menstrual cycles or until a clinically recognized pregnancy occurred. At enrolment both partners filled in a questionnaire on demographic, medical, reproductive, occupational and lifestyle factors. The men provided a semen sample and women a blood sample. From the 430 couples enrolled, PFC concentrations were determined in a subsample of 222 women for whom blood sample was available for analysis.

The PFC analysis

From serum samples, the specific PFCs and their metabolites analyzed comprise PFOS, PFOA, perfluorohexane sulfonic acid (PFHxS), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), *N*-methyl-perfluorooctanoic sulfonamidoacetate (MeFOSAA), *N*-ethyl-perfluorooctane-sulfonamidoacetate (EtFOSAA) and perfluorooctane sulfonamide (FOSA).

The analysis was performed at Environmental Medicine, University of Southern Denmark, Denmark as described by Haug *et al.* (2009), with minor modification. The liquid chromatography–mass spectrometry (LC-MS/MS) system utilized was a Thermo Scientific EQuan MAX system consisting of a TSQ Quantum Ultra with heated-electrospray ionization operated in a negative-ion mode, connected to an Accela high-performance liquid chromatography (HPLC) pump and a CTC autosampler (Thermo Fisher Scientific, San Jose, CA, USA). The separation was performed on a Betasil C8 50 × 3 mm (5 μm) column from Thermo Fischer Scientific (Palo Alto, CA, USA) by the use of a gradient system. The mass transitions, collision energies and tube lens settings were optimized individually for all the compounds. The compounds were quantified by the use of the ^{13}C-isotope-labeled analogs as internal standards (except for PFHxS that was calculated by the use of the internal standard for PFOA). The on-line solid phase extraction of the serum samples was performed on 150-μl serum using the CTC sampler equipped with a double 6-valve VICI switch-valve system. Sample loading and extraction were performed with an extra Accela HPLC pump. The loading pump and the switch valves were controlled from the Excalibur LC-MS/MS software and the extraction was performed on a Betasil C8 10 × 4 mm (5 μm) drop-in guard column (Thermo Fisher Scientific). For PFOA, PFOS, PFHxS, PFNA and PFDA, the within-batch coefficients of variation (CV) were <3% and the between-batch CV were <5.2%. For the metabolites, FOSA, MeFOSAA and EtFOSAA, the within-batch CV were <4.1%, whereas the between-batch CV were <6.8%. The limit of quantification (LOQ) was 0.03 ng/ml for all the reported compounds. Values below the limit were replaced with half of the LOQ (i.e. 0.015 ng/ml) as previously suggested by guidelines (United States Environmental Protection Agency, 2000). The rationale for this procedure is that concentrations may be assumed to be between zero and LOQ and simply excluding cases with values below LOQ from data analysis may bias results (Duval and Karlsson, 2002). Nonetheless, we still acknowledge that this procedure has been argued to bias the results (Schisterman *et al.*, 2006).

The accuracy and reliability of the data are continuously ensured by including quality control samples [e.g. from National Institute of Standards and Technology (NIST)] in each analytical batch of samples, calibration standards, along with reagent and serum blanks. Excellent results were obtained in the regular comparisons scheme organized by the German Society of Occupational Medicine (G-EQUAS). Inspection of the data from the quality assurance samples from NIST-1958 across a period of 3 months in which the reported samples also were analyzed showed the following average bias (measured concentration − target NIST concentration/target NIST concentration) × 100%) for the represented compounds: PFOS: +3%, PFOA: −7%, PFNA: −3% and PFHxS: +3%.

Outcome

TTP was measured in the number of cycles from cessation of birth control to pregnancy or for maximally 6 cycles. Menstrual cycle log books were updated daily during the period at risk. Method of cycle determination has been previously described (Bonde *et al.*, 1998; Jensen *et al.*, 1998). In short, the first cycle of follow-up was defined as the cycle in which birth control was discontinued if more than 10 days had elapsed from discontinuation to the next menstrual bleeding. If not, then the next cycle was considered the first. We had cycle-specific information about the frequency of sexual intercourse and defined a fertile window with at least one sexual intercourse between Days 11 and 20 before the first day of the menstrual cycle, as no couples without sexual intercourse in this window conceived. However, cycle-specific information was missing in 251 cycles and we therefore did not include this information in the analyses but performed sub-analyses after exclusion of 22 cycles without sexual intercourse in the fertile window.

Pregnancy was determined clinically by a physician or a normal pregnancy test and/or by hCG analysis. However, as the hCG sample collection was not complete, for the present study pregnancy was defined as a clinical pregnancy. Subfecundability was in the present study defined as TTP >6 cycles.

Covariates

Age in years was categorized into three age groups (19–24, 25–29 and 30–35). Body weight (kg) and body height (m) were obtained at the clinical visit at baseline. Body mass index (BMI, kg/m^2) was calculated and categorized into three groups (<20, 20–25 and >25). From the questionnaire administered at baseline, smoking habits (never, ever and current), caffeine consumption from tea, coffee and cola (1–149, 150–299, 300–699 and ≥700 mg/d, referring to usage during the recent week), cycle length (20–24, 25–34 and ≥35 days) and contraceptive method until termination (mechanical, intrauterine device and oral contraception) were determined. Similarly, self-reported diseases related to fecundability (females: salpingitis, ovarian cysts, gonorrhea, perforated appendicitis, Crohn's disease, hormonal disorder, amenorrhea or hormonal treatment; males: epididymitis, adult parotitis, gonorrhea, testicular cancer, varicocele, sperm cyst and cryptorchidism) were categorized into a variable (present or not present) for females and males, respectively (Hjollund *et al.*, 1998). Based on the semen sample, sperm concentration (million/ml) was determined. Having a sperm concentration <20 million/ml defined those with oligospermia (no, yes) for the purpose of the study.

Statistical analyses

PFC exposure variables were categorized into groups below and at or above the median. PFC variables were not normally distributed; therefore natural log-transformation was carried out before calculating Pearson correlation coefficients to determine the relationship between PFC variables. Differences in baseline characteristics between those with and without a PFC determination as well as between groups of low and high exposure were analyzed with χ^2 tests. Odds of subfecundability were analyzed using logistic regression. Difference in TTP in low and high PFC exposure was determined by the FR and analyzed using discrete-time survival models (Scheike and Jensen, 1997; Zhu *et al.*, 2009) with complementary log–log link in STATA 11.1 (StataCorp, College Station, TX, USA). To determine the robustness of data, survival models were also carried out with log-transformed and continuous PFC variables. Proportional hazards assumptions were tested using the log-rank goodness-of-fit test based on the Schoenfeld residual including each PFC variable with all covariates. For comparison with results from another Danish study (Fei *et al.*, 2009), survival models were carried out using their quartile cut-points for PFOS and PFOA. We also took into consideration the issue of fertile window by performing analysis excluding cycles without at least one intercourse between Days 11 and 20 before the first day of the period (Kolstad *et al.*, 1999). $P < 0.05$ was determined as statistical significance.

Results

Women without a determination of PFC ($n = 208$) did not have a different TTP, BMI or number of ever smokers compared with those ($n = 222$) with PFC determination. Among the 222 women, the mean age was 26.6 (SE: 0.2) years. Complete follow-up of pregnancy outcome was attained in the present study. Pregnancy was attained in six cycles among 129 out of the 222 couples (58%) and a total of 938 menstrual cycles contributed to the analysis. Among those attaining pregnancy ($n = 129$), 48% became pregnant within the first two cycles, and 16, 15, 13 and 8% attained pregnancy in cycles 3, 4, 5 and 6, respectively.

Baseline characteristics were not associated with PFOS exposure (Table I). There was a statistical significant moderate-to-high correlation (*r*: 0.27–0.85) between log-transformed PFOS and the other log-transformed PFCs. The lowest correlation coefficient observed was between PFOS and EtFOSAA and the highest observed was between PFOS and PFOA. The proportional hazards assumptions were not violated as the *P*-values from the global test were between 0.72 and 0.81. Serum concentrations of PFC did not differ among those becoming pregnant and those who did not (Table II). However, covariates previously found as confounders (maternal age, BMI, smoking, length of menstrual cycle and female diseases affecting fecundability; Jensen *et al.*, 1998) and oligospermia were included in the adjusted models despite not strictly qualifying to be confounders within the present data set to be conservative. The frequency of detection of PFCs ranged from 100 to 81.5% of the samples (Table II).

The odds ratio (OR) of not becoming pregnant within the first six cycles for those with serum concentrations of PFOS and PFOA above the median was 0.96 [95% CI: 0.57–1.64] and 0.96 (95% CI: 0.57–1.64), respectively, compared with those below the median (Fig. 1, Table III). The monthly probability of conceiving (FR) for

Table I Baseline characteristics of the Danish study population by groups below, and at or above, median of PFOS.

	<Median ($n = 111$)	≥Median ($n = 111$)	*P*-value
Female characteristics, *n* (%)			
Age groups (years)			
19–24	31 (27.9)	28 (25.2)	
25–29	71 (64.0)	72 (64.9)	
30–35	9 (8.1)	11 (9.9)	0.84
BMI (kg/m^2)			
<20	23 (20.7)	27 (24.6)[a]	
20–25	65 (58.6)	61 (55.5)	
>25	23 (20.7)	22 (20.0)	0.79
Smoking			
Never	47 (42.3)	60 (54.1)	
Ever	29 (26.1)	16 (14.4)	
Current	35 (31.5)	35 (31.5)	0.07
Caffeine consumption (mg)			
0–149	26 (23.4)	31 (27.9)	
150–299	31 (27.9)	28 (25.2)	
300–699	42 (37.8)	44 (39.6)	
≥700	12 (10.8)	8 (7.2)	0.70
Cycle length (days)			
20–24	2 (1.8)	2 (1.8)[a]	
25–34	86 (78.2)	92 (83.6)	
>35	22 (20.0)	16 (14.6)	0.56
Diseases affecting fecundability			
No	99 (89.2)	100 (90.1)	
Yes	12 (10.8)	11 (9.9)	0.83
Contraceptive method			
Mechanical	77 (69.4)	71 (64.0)	
Intrauterine device	3 (2.7)	3 (2.7)	
Oral contraception	31 (27.9)	37 (33.3)	0.68
Male characteristics, *n* (%)			
Age groups (years)			
19–24	15 (13.5)	15 (13.5)	
25–29	63 (56.8)	70 (63.1)	
30–35	33 (29.7)	26 (23.4)	0.55
Diseases affecting fecundability			
No	101 (91.0)	100 (90.1)	
Yes	10 (9.0)	11 (9.9)	0.82
Oligospermia			
No	99 (89.2)	95 (85.6)	
Yes	12 (10.8)	16 (14.4)	0.42

χ^2 tests were applied to test group differences.
[a]One missing in BMI and two in cycle length.

Table II Concentration of PFC from serum (ng/ml) in female pregnancy planners by subsequent pregnancy status.

	Frequency of detection (% of all)	No pregnancy (n = 93) Median (25th, 75th percentiles)	Pregnancy (n = 129) Median (25th, 75th percentiles)	P-value[a]
PFOS	100	35.75 (27.37, 49.27)	36.29 (30.39, 48.82)	0.29
PFOA	100	5.58 (4.54, 8.02)	5.61 (4.74, 8.12)	0.66
PFHxS	100	1.12 (0.8, 1.64)	1.22 (0.9, 1.83)	0.06
PFNA	100	0.45 (0.36, 0.67)	0.51 (0.38, 0.65)	0.48
PFDA	97.8	0.10 (0.07, 0.14)	0.11 (0.08, 0.14)	0.45
MeFOSAA	99.5	0.45 (0.26, 0.87)	0.39 (0.23, 0.69)	0.27
EtFOSAA	99.5	2.12 (1.45, 3.17)	1.79 (1.2, 3.04)	0.27
FOSA	81.5	0.10 (0.05, 0.19)	0.11 (0.04, 0.16)	0.48

PFC, perfluorinated compounds; PFOA, perfluorooctanoic acid; PFOS perfluorooctane sulfonic acid; PFHxS, perfluorohexane sulfonic acid; PFDA, perfluorodecanoic acid; PFNA, perfluorononanoic acid; MeFOSAA, *N*-methyl-perfluorooctanoic sulfonamidoacetate; EtFOSAA, *N*-ethyl-perfluorooctane-sulfonamidoacetate; FOSA, perfluorooctane sulfonamide.
[a]Wilcoxon rank-sum test for difference between groups.

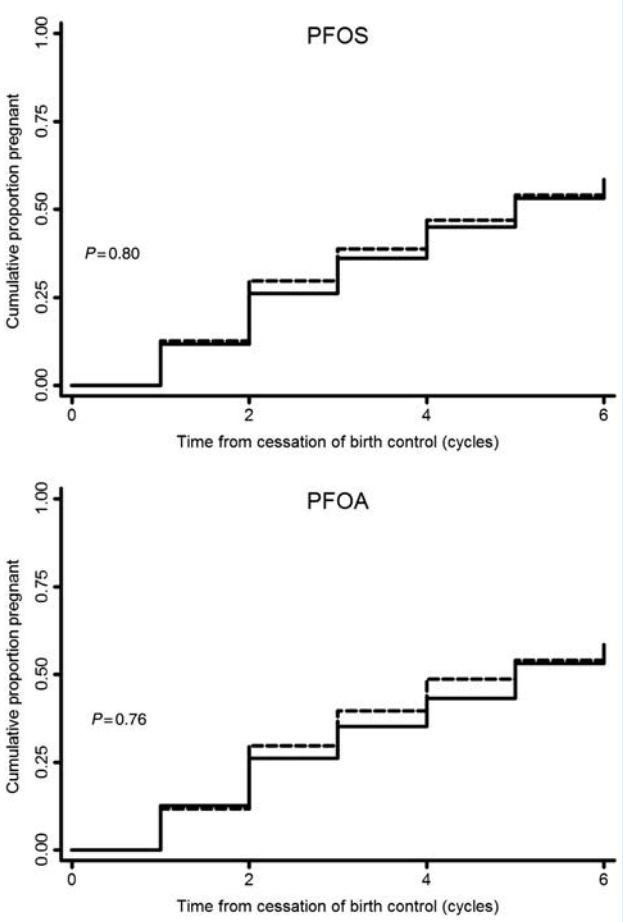

Figure 1 Cumulative proportion of Danish women pregnant by exposure to specific perfluorinated compounds. Kaplan–Meier curves. Dashed line describes PFC < median. Solid line describes PFC ≥ median. *P*-value describes the difference between curves tested using a discrete-time survival model with the complementary log–log link. PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonic acid.

those with values above the median concentrations of PFOS and PFOA was 1.05 (95% CI: 0.74–1.48) and 1.05 (95% CI: 0.74–1.49), respectively, compared with those below the median (Table III). Similarly, results for the remaining six PFCs (PFHxS, PFNA, PFDA, EtFoSAA, MeFOSAA and FOSA) also did not deviate from the null hypothesis of no difference in TTP with increased PFC exposure. Adjusting for maternal age, BMI, smoking, length of menstrual cycle, female diseases affecting fecundability and oligospermia did not alter ORs and FRs markedly from the unadjusted analysis for all eight PFCs. To facilitate interpretation of the magnitude of the estimates presented in Table III, the presence of either female diseases affecting fecundability or oligospermia resulted in FRs of 0.46 (95% CI: 0.23–0.94) and 0.34 (95% CI: 0.17–0.67), respectively, in the adjusted model of PFOS and, thus, resulting in the expected direction of the FRs. Using the PFC concentrations as a continuous independent variable in the analysis instead of the dichotomized independent variable altered the estimates for FRs of PFOS, PFOA and FOSA to some extent. However, the FRs were still statistically insignificant and the CIs largely overlapped with those from prior analyses of the dichotomized PFC variables. Excluding the cycles in which the couple did not have intercourse between Days 11 and 20 before menstrual bleeding (cycles = 22) did not change the estimates (data not shown).

Restricting our analysis to only those who became pregnant resulted in FRs of 1.16 (95% CI: 0.78–1.74) and 1.10 (95% CI: 0.75–1.62) for those above the median serum concentrations of PFOS and PFOA, respectively. From the total sample, performing the analysis while applying the same cut-points as used in the study of Fei *et al*. (2009; PFOS: low = ≤26 ng/ml, high = ≥43.3 ng/ml; PFOA: low≤ 3.91 ng/ml, high = ≥ 6.97 ng/ml) and then comparing the higher concentrations with the lowest concentrations (ref. group) resulted in an FR of 1.60 (95% CI: 0.90–2.86) for PFOS and an FR of 1.11 (95% CI: 0.62–1.96) for PFOA. To facilitate interpretation of the magnitude of the estimates in the latter analysis, the presence of either female diseases affecting fecundability or presence of oligospermia resulted in FRs of 0.31 (95% CI: 0.11–0.87) and 0.37 (95% CI: 0.15–0.87), respectively, when controlling for the other variables in the adjusted model of PFOS.

Table III OR of subfecundability and FR for PFCs below, or at and above, their median concentrations (ng/ml).

	Pregnancies (*n*)	Subfecundability (%)	OR (95% CI) of subfecundability		FR (95% CI)	
			Unadjusted	Adjusted[a]	Unadjusted	Adjusted[a]
PFOS						
<36.28	64	42.3	1	1	1	1
≥36.28	65	41.4	0.96 (0.57–1.64)	0.98 (0.54–1.77)	1.05 (0.74–1.48)	1.03 (0.72–1.47)
Log-transformed and continuous					1.27 (0.85–1.90)	1.39 (0.92–2.09)
PFOA						
<5.602	64	42.3	1	1	1	1
≥5.602	65	41.4	0.96 (0.57–1.64)	1.21 (0.67–2.18)	1.05 (0.75–1.49)	0.92 (0.65–1.31)
Log-transformed and continuous					1.11 (0.74–1.66)	1.18 (0.78–1.78)
PFHxS						
<1.162	60	46.0	1	1	1	1
≥1.162	69	37.8	0.72 (0.42–1.22)	0.67 (0.37–1.20)	1.28 (0.90–1.81)	1.29 (0.90–1.83)
Log-transformed and continuous					1.34 (1.04–1.71)	1.33 (1.01–1.75)
PFNA						
<0.478	61	45.1	1	1	1	1
≥0.478	68	38.7	0.77 (0.45–1.32)	0.67 (0.37–1.25)	1.17 (0.83–1.66)	1.25 (0.87–1.79)
Log-transformed and continuous					1.07 (0.80–1.44)	1.17 (0.88–1.54)
PFDA						
<0.1017	60	46.0	1	1	1	1
≥0.1017	69	37.8	0.72 (0.42–1.22)	0.61 (0.33–1.12)	1.24 (0.87–1.75)	1.40 (0.96–2.03)
Log-transformed and continuous					1.05 (0.81–1.34)	1.15 (0.89–1.49)
MeFOSAA						
<0.414	69	37.8	1	1	1	1
≥0.414	60	46.0	1.40 (0.82–2.38)	1.23 (0.68–2.20)	0.78 (0.55–1.11)	0.90 (0.63–1.30)
Log-transformed and continuous					0.93 (0.77–1.11)	0.99 (0.82–1.18)
EtFOSAA						
<1.91	67	39.1	1	1	1	1
≥1.91	62	44.6	1.26 (0.74–2.14)	1.53 (0.85–2.77)	0.90 (0.64–1.28)	0.80 (0.56–1.15)
Log-transformed and continuous					0.84 (0.67–1.07)	0.79 (0.62–1.00)
FOSA						
<0.098	62	44.1	1	1	1	1
≥0.098	67	39.6	0.83 (0.49–1.42)	0.81 (0.45–1.46)	1.14 (0.81–1.62)	1.23 (0.85–1.76)
Log-transformed and continuous					0.95 (0.81–1.11)	1.01 (0.86–1.18)

OR, odds ratio; FR, fecundability ratio; CI, confidence interval; PFC, perfluorinated compounds; PFOA, perfluorooctanoic acid; PFOS perfluorooctane sulfonic acid; PFHxS, perfluorohexane sulfonic acid; PFDA, perfluorodecanoic acid; PFNA, perfluorononanoic acid; MeFOSAA, N-methyl-perfluorooctanoic sulfonamidoacetate; EtFOSAA, N-ethyl-perfluorooctane-sulfonamidoacetate; FOSA, perfluorooctane sulfonamide.

[a]Adjusted for female age, BMI, smoking, length of menstrual cycle, female diseases affecting fecundability, oligospermia (1, case excluded because of missing BMI; 2, because of missing in length of menstrual cycle corresponding to a total loss of nine cycle months). *Note*: FR >1 describes a better chance of getting pregnant. FR in lines of log-transformed and continuous PFC variables is a separate survival analysis, and describes the increased or decreased risk per one unit increase in the PFC concentration.

Discussion

The present study evaluated the association of female serum concentrations of eight different PFCs with TTP in first-pregnancy planners. The results failed to show a clear association between any of the measured PFCs and TTP. Adjusting for age and other factors known to affect both female and male fecundability did not alter the findings.

Consistently for all measured PFCs, the results of the present study are not in concordance with previously observed results which suggested that higher concentrations of both PFOA and PFOS were associated with longer TTP among pregnant women (Fei *et al.*, 2009). However, Fei *et al.* (2009) differed in study design, sample size, timing of blood sample collection and control for semen quality. Most importantly, their study population comprised pregnant women of whom many had previous pregnancies. Our study population included only first-pregnancy planners without any knowledge of their reproductive capability, with 58% achieving pregnancy within six cycles. Also, maternal age was slightly higher in the study of Fei *et al.* (2009) compared with our study; however, both studies include maternal age as covariate in the models.

Regarding the difference between an exclusively null-parity and a mixed parity design, previous pregnancy will result in a lower serum concentration as PFCs pass the placenta and are excreted in human milk (Thomsen *et al.*, 2010; Liu *et al.*, 2011). This bias (reverse causality) will particularly affect highly fertile women, who are likely to have a shorter TTP to second pregnancy and consequently also lower PFCs levels. Hence, although the biological plausibility is uncertain, this factor might explain the divergence of results between Fei *et al.* (2009) and our study. Reverse causality is supported by Whitworth *et al.* (2011) based on data from a Norwegian study, showing an association between PFCs and subfecundity among parous but not among nulliparous women.

A mechanism through which PFCs may increase TTP is not clear but it has been suggested that PFCs may affect the hypothalamus–pituitary–ovarian regulation whereby, for example, delayed ovulation occurs (Fei *et al.*, 2009). We took this possible mechanism into consideration by including the length of menstrual cycle as a covariate, although this adjustment did not change the results. Furthermore, mouse models have shown increasing embryo resorption and prenatal loss with increasing PFOA exposure dosage (Lau *et al.*, 2006). Although PFC doses demonstrating reproductive toxicity in animal studies are much higher than those of human experience, assuming the effect would be similar, higher numbers of sub-clinical pregnancies and abortions would be found in groups with higher serum concentrations of PFCs. This may also constitute a difference between the present study and that of Fei *et al.*'s (2009), where women were included when they were already pregnant. However, we found no differences in the proportion of early hCG-positive pregnancies among women with different PFC levels.

Strengths of this study include the prospective design, whereby bias introduced by unplanned pregnancies as well as recall bias resulting from difficulties of attaining pregnancy was avoided. Also, information bias related to the outcome was reduced as TTP was carefully followed through monthly diaries. Additionally, blood samples for PFC determinations were collected when birth control was discontinued, serum concentrations carefully determined and a high frequency of detection related to the low LOQ was attained for the PFCs quantified. Furthermore, the PFOS and PFOA concentrations in the present study were similar to those observed in the other Danish study (Fei *et al.*, 2009). In detail, in the present study and that of Fei *et al.* (2009), the median PFOS concentration was 36.3 ng/ml compared with 33.7 ng/ml and the median PFOA concentration was 5.6 ng/ml compared with 5.3 ng/ml, respectively. Therefore, we believe that information bias related to exposure is negligible. To extend this comparison to data outside of Europe, PFOS and PFOA concentrations from 20- to 39-year-old participants in the National Health and Nutrition Examination Survey 1999–2000 were slightly lower than from our participants (Calafat *et al.*, 2007), which may be a result of their data collection being in 1999–2000 as opposed to 1992–1995 in our study. Unfortunately, we do not have information on male PFC exposure. Assuming the mechanism through which this would contribute to subfecundity could be through poor semen quality; this may have been partly adjusted for in our analytic models.

Different from the stratification into above or at or below the median in our study, Fei *et al.* divided PFC exposure into quartiles (Fei *et al.*, 2009), which again could cause difference in results. Their finding of the detrimental effect of PFOA and PFOS may possibly be driven in particular by a high fecundability in the lowest quartile. However, analysis using the same cut-points in our data did not support the findings of Fei *et al.* (2009) and neither when we applied the log-transformed continuous variables in the survival models. Also, as they included only those known to be pregnant, we performed analysis restricted to only those who became pregnant. Although the direction of the estimates do not concur, it is prudent to recognize that the CIs for the effect estimates of the two studies overlap.

Although our sample size was smaller, the present study had the power to detect differences of the magnitude suggested by Fei *et al.* (Altman, 1991; Fei *et al.*, 2009). Nevertheless, our study had a shorter follow-up period and therefore less time compared with Fei *et al.* (2009) to determine events. Fewer events may also be caused by a possible fraction of infertile couples among eligible couples, despite our attempt to exclude couples with previous unsuccessful reproductive experience from the sample in the design phase and the adjustment for female risk factors affecting fecundability as well as semen quality. Furthermore, as the percentage attaining pregnancy within the two first cycles was similar between the present study (48%) and that of Fei *et al.* (2009; 50%), it seems that the risk of being underpowered to assess significant FR related to concerns of all becoming pregnant in the first two cycles is minor.

The participants in the present study are likely a selected group. The question is the extent to which this affects the results. TTP was not related to having serum available for PFC determination within the overall sample of 430 couples. Nevertheless, those not participating at all may possibly have higher PFC serum concentrations. Assuming that PFC is in fact associated with longer TTP, it is possible that we underestimated the true effect of PFC. However, the effect of possible selection bias is reduced by the choice of prospective design in our study, in contrast to the retrospective design including only those attaining pregnancy (Weinberg *et al.*, 1994; Fei *et al.*, 2009). Among couples at risk of pregnancy (e.g. not using contraception) there is, overall, a wide heterogeneity in fecundability (Weinberg *et al.*, 1994) and by including couples with an unknown fecundability probability from the start, as in the present study, we may have achieved a

more accurate estimate of the effect of PFC on TTP (Weinberg *et al.*, 1994).

Given the long-term storage of serum samples (~15 years), it is possible that integrity of the serum samples for PFC analysis is reduced. Unfortunately, to our knowledge, there are no studies on the degradation of PFCs in long-term stored frozen serum samples. However, it may in fact be a minor problem as the major issue of PFCs is their persistency to degradation (Fromel and Knepper, 2010) and degradation is probably not related to TTP. Finally, our inclusion of confounders which are not strictly confounders (e.g. menstrual cycle length) could potentially bias the estimates of the effect of PFC on TTP. However, as the crude and adjusted estimates within the present study were essentially no different, this is considered negligible.

In conclusion, the present results provided no support for the hypothesis that exposure to different PFCs substantially affects fecundability as measured by TTP in a general population of couples planning their first-time pregnancy. Nevertheless, the precautionary principle should be applied and further limitations on the production of PFCs could be continued until further studies have been carried out.

Acknowledgements

We are indebted to the trade union officials for support and we thank the technicians from the laboratories for performing the exposure and semen analysis, and the study participants.

Authors' roles

S.V. was involved in the study concept, analysis and interpretation of the data, and drafting of the manuscript. F.N. was involved in the analysis of PFC in serum samples, interpretation of the data and revision of the manuscript. A.M.A. contributed to acquisition of participants and data, interpretation of data and revision of the manuscript. N.H.H. contributed to acquisition of participants and data, interpretation of the data and revision of the manuscript. P.G. contributed to interpretation of the data and revision of the manuscript. H.R.A. contributed to interpretation of the data and revision of the manuscript. T.K.J. contributed to study concept, acquisition of participants and data, guided analysis, interpretation of data and revision of the manuscript.

Funding

This study was supported by a grant from the Aarhus University Research Foundation (J nr 1994-7430-1) with an additional support from the Danish Medical Research Council (J nr 12-2042-1), the Danish Medical Health Insurance Foundation (J nr 11/236-93 and J nr 11/243-91), the Danish Research Council (Grant 09-065175) and the Kirsten and Freddy Johansen Fund (grant 95-103-21224).

Conflict of interest

None declared.

References

Altman DG. *Practical Statistics for Medical Research*, 1st edn. Boca Raton, London: Chapman and Hall/CRC, 1991, 456–459.

Bonde JP, Hjollund NH, Jensen TK, Ernst E, Kolstad H, Henriksen TB, Giwercman A, Skakkebaek NE, Andersson AM, Olsen J. A follow-up study of environmental and biologic determinants of fertility among 430 Danish first-pregnancy planners: design and methods. *Reprod Toxicol* 1998;**12**:19–27.

Calafat AM, Wong LY, Kuklenyik Z, Reidy JA, Needham LL. Polyfluoroalkyl chemicals in the U.S. population: data from the National Health and Nutrition Examination Survey (NHANES) 2003–2004 and comparisons with NHANES 1999–2000. *Environ Health Perspect* 2007;**115**:1596–1602.

Duval V, Karlsson MO. Impact of omission or replacement of data below the limit of quantification on parameter estimates in a two-compartment model. *Pharm Res* 2002;**19**:1835–1840.

EFSA (European Food Safety Authority). Opinion of the scientific panel on contaminants in the food chain on perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts. *EFSA J* 2008;**653**:1–131.

Fei C, Olsen J. Re: serum levels of perfluorooctanoic acid and perfluorooctane sulfonate and pregnancy outcome. *Am J Epidemiol* 2010;**171**:131–132.

Fei C, McLaughlin JK, Tarone RE, Olsen J. Fetal growth indicators and perfluorinated chemicals: a study in the Danish National Birth Cohort. *Am J Epidemiol* 2008;**168**:66–72.

Fei C, McLaughlin JK, Lipworth L, Olsen J. Maternal levels of perfluorinated chemicals and subfecundity. *Hum Reprod* 2009;**24**:1200–1205.

Fromel T, Knepper TP. Biodegradation of fluorinated alkyl substances. *Rev Environ Contam Toxicol* 2010;**208**:161–177.

Halldorsson TI, Fei C, Olsen J, Lipworth L, McLaughlin JK, Olsen SF. Dietary predictors of perfluorinated chemicals: a study from the Danish National Birth Cohort. *Environ Sci Technol* 2008;**42**:8971–8977.

Haug LS, Thomsen C, Becher G. A sensitive method for determination of a broad range of perfluorinated compounds in serum suitable for large-scale human biomonitoring. *J Chromatogr A* 2009;**1216**:385–393.

Haug LS, Huber S, Becher G, Thomsen C. Characterisation of human exposure pathways to perfluorinated compounds—comparing exposure estimates with biomarkers of exposure. *Environ Int* 2011;**37**:687–693.

Hjollund NH, Bonde JP, Jensen TK, Henriksen TB, Kolstad HA, Ernst E, Giwercman A, Pritzl G, Skakkebaek NE, Olsen J. A follow-up study of male exposure to welding and time to pregnancy. *Reprod Toxicol* 1998;**12**:29–37.

Jensen TK, Hjollund NH, Henriksen TB, Scheike T, Kolstad H, Giwercman A, Ernst E, Bonde JP, Skakkebaek NE, Olsen J. Does moderate alcohol consumption affect fertility? Follow up study among couples planning first pregnancy. *BMJ* 1998;**317**:505–510.

Kolstad HA, Bonde JP, Hjollund NH, Jensen TK, Henriksen TB, Ernst E, Giwercman A, Skakkebaek NE, Olsen J. Menstrual cycle pattern and fertility: a prospective follow-up study of pregnancy and early embryonal loss in 295 couples who were planning their first pregnancy. *Fertil Steril* 1999;**71**:490–496.

Lau C, Thibodeaux JR, Hanson RG, Narotsky MG, Rogers JM, Lindstrom AB, Strynar MJ. Effects of perfluorooctanoic acid exposure during pregnancy in the mouse. *Toxicol Sci* 2006;**90**:510–518.

Liu J, Li J, Liu Y, Chan HM, Zhao Y, Cai Z, Wu Y. Comparison on gestation and lactation exposure of perfluorinated compounds for newborns. *Environ Int* 2011;**37**:1206–1212.

Olsen GW, Butenhoff JL, Zobel LR. Perfluoroalkyl chemicals and human fetal development: an epidemiologic review with clinical and toxicological perspectives. *Reprod Toxicol* 2009;**27**:212–230.

Rachootin P, Olsen J. Prevalence and socioeconomic correlates of subfecundity and spontaneous abortion in Denmark. *Int J Epidemiol* 1982;**11**:245–249.

Scheike TH, Jensen TK. A discrete survival model with random effects: an application to time to pregnancy. *Biometrics* 1997;**53**:318–329.

Schisterman EF, Vexler A, Whitcomb BW, Liu A. The limitations due to exposure detection limits for regression models. *Am J Epidemiol* 2006; **163**:374–383.

Steenland K, Fletcher T, Savitz DA. Epidemiologic evidence on the health effects of perfluorooctanoic acid (PFOA). *Environ Health Perspect* 2010; **118**:1100–1108.

Thomsen C, Haug LS, Stigum H, Froshaug M, Broadwell SL, Becher G. Changes in concentrations of perfluorinated compounds, polybrominated diphenyl ethers, and polychlorinated biphenyls in Norwegian breast-milk during twelve months of lactation. *Environ Sci Technol* 2010;**44**:9550–9556.

Trier X, Granby K, Christensen JH. Polyfluorinated surfactants (PFS) in paper and board coatings for food packaging. *Environ Sci Pollut Res Int* 2011;**18**:1108–1120.

Trudel D, Horowitz L, Wormuth M, Scheringer M, Cousins IT, Hungerbuhler K. Estimating consumer exposure to PFOS and PFOA. *Risk Anal* 2008;**28**:251–269.

United States Environmental Protection Agency. *Guidance for Data Quality Assessment. Practical Methods for Data Analysis (EPA QA/G-9). EPA/600/R-96/084*. Washington, DC, USA: In Office of Environmental Information, 2000.

Weinberg CR, Baird DD, Wilcox AJ. Sources of bias in studies of time to pregnancy. *Stat Med* 1994;**13**:671–681.

Whitworth KW, Haug LS, Baird DD, Becher G, Hoppin JA, Skjaerven R, Thomsen C, Eggesbo M, Travlos G, Wilson R *et al.* Perfluorinated compounds and subfecundity in pregnant women. *Epidemiology* 2011. doi:10.1097/EDE.0b013e31823b5031.

Xiao F, Simcik MF, Gulliver JS. Partitioning characteristics of perfluorooctane sulfonate between water and foods. *Arch Environ Contam Toxicol*, 2012;**62**:42–48.

Zhu JL, Obel C, Basso O, Bech BH, Henriksen TB, Olsen J. Handedness and time to pregnancy. *Epidemiology* 2009;**20**:52–55.