Human Reproduction, Vol.28, No.3 pp. 599–608, 2013
Advanced Access publication on December 18, 2012 doi:10.1093/humrep/des425

human reproduction

ORIGINAL ARTICLE *Andrology*

PFOS (perfluorooctanesulfonate) in serum is negatively associated with testosterone levels, but not with semen quality, in healthy men

Ulla Nordström Joensen[1,*], Bruno Veyrand[2], Jean-Philippe Antignac[2], Martin Blomberg Jensen[1], Jørgen Holm Petersen[1], Philippe Marchand[2], Niels Erik Skakkebæk[1], Anna-Maria Andersson[1], Bruno Le Bizec[2], and Niels Jørgensen[1]

[1]University Department of Growth and Reproduction, Rigshospitalet, Section 5064, Blegdamsvej 9, Copenhagen DK-2100, Denmark
[2]LUNAM Université, Oniris, Laboratoire d'Etude des Résidus et Contaminants dans les Aliments (LABERCA), USC INRA 1329, Nantes F-44307, France

*Correspondence address. Tel: +45-3545-5064; Fax: +453545-6054; E-mail: ulla.nordstroem.joensen@rh.regionh.dk

Submitted on July 27, 2012; resubmitted on November 7, 2012; accepted on November 12, 2012

STUDY QUESTION: Is exposure to perfluorinated compounds (PFCs) associated with testicular function (reproductive hormone levels and semen quality) in healthy men?

SUMMARY ANSWER: PFOS levels were significantly negatively associated with serum testosterone (total and calculated free), but not with any other reproductive hormones or semen quality.

WHAT IS KNOWN ALREADY: In animals, some PFCs have endocrine disrupting potential, but few studies have investigated PFCs in relation to human testicular function. Previously, we and others have observed a negative association between serum PFC levels and sperm morphology. The potential associations with reproductive hormones remain largely unresolved.

STUDY DESIGN, SIZE, DURATION: A cross-sectional study of 247 men was conducted during 2008–2009.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Healthy men from the general population, median age of 19 years, gave serum and semen samples. Serum samples were analysed for total testosterone (T), estradiol (E), sex hormone-binding globulin (SHBG), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and inhibin-B and 14 PFCs, including perfluorooctanesulfonate (PFOS). Semen samples were analysed according to the WHO criteria.

MAIN RESULTS AND THE ROLE OF CHANCE: PFOS levels were negatively associated with testosterone (T), calculated free testosterone (FT), free androgen index (FAI) and ratios of T/LH, FAI/LH and FT/LH. Other PFCs were found at lower levels than PFOS and did not exhibit the same associations. PFC levels were not significantly associated with semen quality. PFOS levels in these samples collected in 2008–2009 were lower than in our previous study of men participating in 2003.

LIMITATIONS, REASONS FOR CAUTION: Results were robust to adjustment for relevant confounders; however, the possibility of chance associations due to multiple testing or effects of uncontrolled confounding cannot be ruled out.

WIDER IMPLICATIONS OF THE FINDINGS: Our previous findings of decreased sperm morphology in the most highly PFC exposed men were not replicated, possibly due to a lack of highly exposed individuals; however, a recent independent study also did corroborate such an inverse association. The negative association between serum PFOS and testosterone indicates that testosterone production may be compromised in individuals with high PFOS exposure.

STUDY FUNDING/COMPETING INTEREST(S): The authors received financial support from the European Commission (DEER, FP7-2007-212844), the Danish Agency for Science, Technology and Innovation (grant nos. 27107068 and 09-067180), Rigshospitalet (grant no. 961506336), the University of Copenhagen, the Danish Ministry of Health and the Danish Environmental Protection Agency (MST-621-00013), and Kirsten and Freddy Johansen Foundation (grant no. 95-103-72087). The funding organizations played no role in

the design and conduct of the study, in collection, management, analysis and interpretation of the data; or in the presentation, review or approval of the manuscript. The authors declare that they have no competing financial interests.

Key words: PFC / PFOS / PFOA / testosterone / semen quality

Introduction

Perfluorinated compounds (PFCs) are synthetic chemicals with lipophobic and hydrophobic properties. The compounds are widely used for industrial purposes (lubricants, surfactants) and consumer products such as non-stick cookware, clothing, carpets and paper (Kissa, 2001). Food packaging may be a source of perfluorooctanesulfonate (PFOS) and perfluoro-*n*-octanoic acid (PFOA) migration into food (Begley *et al*., 2008), as well as other fluorinated compounds that can biotransform into PFOS and PFOA (Trier *et al*., 2011). Some PFCs have been found to be persistent and subject to bioaccumulation, and humans and wildlife are today exposed globally to these emerging substances (Kannan *et al*., 2004). PFOA is suspected to have endocrine disrupting potential in addition to carcinogenic and immunotoxic effects (Steenland *et al*., 2010; White *et al*., 2011). PFOS and PFOA are similar compounds, but there is less toxicological data on possible reproductive effects of PFOS.

Few epidemiological studies have investigated PFC exposure in relation to testicular function. In a study of 105 healthy men investigated in 2003, we observed that those with high serum levels of PFOS and PFOA combined had fewer normal spermatozoa in their ejaculate than those with low PFOS–PFOA levels (Joensen *et al*., 2009). Associations between other measures of semen quality and PFOS-PFOA levels were also generally negative but not statistically significant. There was a tendency towards lower total testosterone (total T), free androgen index (FAI) and inhibin-B in the highest PFOS–PFOA exposure group, with no change in LH and FSH, but these associations also were not statistically significant. Subsequently, a study of serum PFOS and PFOA levels in 256 American men from an infertility clinic reported a positive correlation between serum LH and both PFOS and PFOA, but no association with testosterone, sperm concentration or motility. Sperm morphology was not assessed, and correlations between reproductive hormones and PFCs were not adjusted for confounders (Raymer *et al*., 2012). A study of partners of pregnant women in three populations (199 men from Greenland, 197 men from Poland and 208 men from Ukraine) showed a positive association between the serum PFOA concentration and sperm DNA damage as well as serum sex hormone-binding globulin (SHBG) levels in men from Greenland, who had the highest PFC exposure, but the associations were not observed across populations. Also, no associations were found between serum PFOS levels and reproductive hormones or any measure of sperm DNA integrity in that study (Specht *et al*., 2012). However in the same population, the authors reported a 35% reduction in sperm morphology in the highest tertile of PFOS exposure when compared with the lowest tertile (Toft *et al*., 2012).

Two previous studies of men occupationally exposed to PFOA in the same production plant have reported diverging results. The first study of 115 employees reported that PFOA was negatively associated with serum testosterone and free testosterone, and positively associated with estradiol, but not with LH (Gilliland, 1992). The second study (123 men) reported no such associations (Olsen *et al*., 1998).

Exposure of adult male rats to PFOA reduces serum testosterone levels and increased estradiol levels, which may partly explain earlier findings of Leydig cell hyperplasia and/or adenomas in testes of exposed animals (Cook *et al*., 1992; Biegel *et al*., 1995). The mechanism of action of PFCs on testicular function is still largely unknown, but the sparse evidence that does exist points towards a non-specific inhibition of androgen secretion on a testicular level (Shi *et al*., 2007; Zhao *et al*., 2010).

In this study, we aimed to investigate the associations between serum PFC concentrations and reproductive hormones and semen quality in a larger unselected group of healthy men.

Materials and Methods

Study population

In 2008–2009, 247 healthy young Danish men from the general population of Denmark were randomly selected among men participating in an on-going study surveying semen quality (Jørgensen *et al*., 2002). The men were approached when going to a medical examination compulsory for all young Danish men being considered for military service. The basic study details have previously been described (Ravnborg *et al*., 2011). Participation rate was ~30%, which is higher than other population-based semen quality studies (Jørgensen *et al*., 2002). The men underwent a physical examination (including the assessment of testis size by palpation and ultrasound), provided a semen sample and had a blood sample drawn, in most cases all within 1 h. Semen samples were produced by masturbation in a room adjacent to the semen laboratory. The men were instructed to abstain from ejaculation at least 48 h prior to their appointment. The ejaculation abstinence period and time of blood sampling were recorded. All semen and blood samples were collected between 8.40 a.m. and 11.50 a.m. Blood samples were drawn from the cubital vein and the serum was stored at −20°C until chemical analysis. All serum and semen analyses were performed blind, i.e. without access to any information regarding the subjects.

The men handed in a questionnaire including the information on lifestyle and medical history. Responses were reviewed with the participant to clarify missing or ambiguous information. Ethnicity was deduced from self-reported country of birth of the participant and his parents. Basic characteristics of the 247 men included in this study can be seen in Table I. Reproductive hormone levels and semen quality parameters are shown in Table II.

PFC analyses

Serum samples were analysed in single evaluations for the multi-residue detection of 14 PFCs (Table III). Sample preparation consisted of an alkaline digestion using potassium hydroxide (KOH) followed by an extraction step by solid phase extraction (SPE) with a polymeric phase Oasis HLB stationary phase and then a purification step by SPE on a carbon graphitized phase envicarb cartridge. Beforehand, in a polypropylene tube, 3 ml KOH (0.1 M in methanol) was added to 1 ml of serum previously fortified with

Table I Basic characteristics of the study population ($n = 247$).

	Mean ± SD	Median (5, 95 pct)	n (%)
Age (years)	19.6 ± 1.4	19.2 (18.4, 22.4)	
BMI (kg/m^2)	22.9 ± 2.8	22.5 (19.2, 28.3)	
Ejaculation abstinence (hours)	73 ± 54	62 (36, 123)	
Previous diseases			
Cryptorchidism[a]			8 (3.2)
Sexually transmitted disease[b]			38 (15)
Varicocele[c]			24 (9.7)
Inguinal hernia			15 (6.0)
Good or very good general health[d]			220 (89)
Medication within last 3 months[e]			37 (15)
>21 units of alcohol last week			74 (30)
Current smoker			104 (42)
Ethnicity			
Danish			192 (78)
Other European			20 (8.1)
Middle Eastern			10 (4.0)
African			4 (1.6)
Asian			5 (2.0)
Latin American			4 (1.6)
Inuit			1 (0.4)
Not reported			11 (4.5)

[a]Not born with both testicles in the scrotum (includes spontaneous descent, treated cases or still cryptorchid).
[b]Included chlamydia, condylomas, genital herpes and/or gonorrhoea.
[c]Varicocele diagnosed previously or on the day of participation.
[d]Question was 'How would you describe your own health? Very good, good, fair or poor'.
[e]11 men had taken medication for skin conditions, 10 systemic antibiotics, 2 analgesics, 9 asthma/allergy medication and 5 other medication.

1 ng of the internal standard mixture. The sample was thoroughly vortex-mixed for 1 min, and left to digest overnight at room temperature. The pH was then adjusted to neutral with 100 μl of glacial acetic acid before centrifugation for 10 min at 4000 rpm. The extract was then evaporated to ~1 ml under a gentle nitrogen stream in a dry bath at 45°C and reconstituted with 4 ml 0.1 M formic acid. The sample was loaded onto the Oasis HLB cartridge preconditioned with 10 ml MeOH and 10 ml 0.1 M formic acid. The cartridge was washed with 5 ml 0.1 M formic acid and 5 ml of MeOH/0.1 M formic acid (50/50, v/v) before elution of the target compounds with 6 ml MeOH/ammonium hydroxide (99/1, v/v). The extract was reduced to 1 ml prior to application on an envicarb cartridge preconditioned with 10 ml MeOH. Then the target analytes were eluted with 6 ml MeOH/glacial acetic acid (80/1; v/v). Finally, the extract was evaporated to dryness and reconstituted in 200 μL MeOH/water (30/70, v/v) with fluorometholone as an external standard. The method has been validated according to the current European analytical criteria and performances have been found fit-for-purpose. Limits of detection (LODs) and limits of quantification (LOQs) ranged from 0.01 to 0.50 ng/ml and from 0.03 to 1.5 ng/ml, respectively, depending on the target substance (Table III). The linearity was assessed on the basis of nine calibration levels for each analyte over the 0.1–50 ng/ml concentration range. Coefficients of determination (R^2) better than 0.990 were found for all analytes. The method accuracy was controlled using a certified reference material (NIST SRM 1957) and through the participation in an international ring test.

The measurement system used included a 1200 series HPLC pump (Agilent, Palo Alto, CA, USA) equipped with a binary low-pressure mixing LC pump (G1312B), with a built-in vacuum degasser (G1379B), a 50-μl loop injection, a temperature controlled autosampler (G1367D) and a column oven (G1316B). The system was fitted with a reverse phase column Gemini C18 (3 μm, 50 × 2.0 mm) equipped with a guard column (3 μm, 10 × 2.0 mm) (Phenomenex, Torrance CA, USA). The mobile phase consisted of methanol (Solvent A) and ammonium acetate 20 mM (Solvent B). The elution gradient started with 30% A for 2 min, followed by a 7-min linear gradient to 100%, then a 5-min hold at 100%, and returned back to 30% in 3 min. The flow rate was 0.6 ml/min and the injection volume was set at 20 μl. The column heater was used to ensure a stable column temperature of 40°C. The HPLC system was interfaced with a linear ion trap coupled to an orbital trap (LTQ-Orbitrap^{TM}) instrument (Thermo Scientific, Germany) operating in negative electrospray ionisation mode. Mass spectra were acquired in full scan mode from m/z 200 to 900 using a mass resolution of 30 000 FWHM at 400 m/z, in the centroid mode. Quantitative sample analysis was performed using extracted mass chromatograms from full scan recording using m/z values listed in Table III (typical mass tolerance of 0.05 uma). The following mass spectrometer parameters were applied: capillary voltage was set at −14 V, source voltage at 4 kV and capillary temperature at 280°C. Nitrogen was used as sheath and auxiliary gas at flow rates of 40 and 10 (arbitrary unit), respectively.

Inter-laboratory comparison with PFC concentrations measured in a previous study

PFC levels in samples collected in 2003 from 105 men were analysed at the Danish National Environmental Research Institute (NERI) as previously described (Joensen *et al.*, 2009). Those 105 men were selected from a total of 500 participants in 2003 based on serum testosterone levels (53 men with high testosterone levels and 52 with low testosterone levels). Remaining serum was available from 25 of the 105 men, and these samples were re-analysed at LABERCA (ONIRIS, France) for inter-laboratory comparison. Results for PFHxS, PFOS, PFOA, PFNA and PFDA were available from both laboratories for these 25 samples (Supplementary data).

Reproductive hormone analyses

Thawed samples were analysed in June 2010. Levels of FSH, LH and SHBG were measured by time-resolved fluoroimmunoassay (Autodelfia, Wallac, Turku, Finland). Total T and E were determined by radioimmunoassay (Coat-a-Count Total Testosterone, Siemens, Los Angeles, USA and Pantex direct ^{125}I, Santa Monica, USA, respectively). Inhibin-B was determined by a double antibody enzyme-immunometric assay using kit material from the Inhibin-B genII assay (Beckman Coulter, USA), together with an in-house recombinant inhibin-B standard. Intra- and inter-assay coefficients of variation for FSH, LH and SHBG were <6%, and CVs for total T were <10%. Intra-and inter-assay CVs for estradiol were 8 and 13%, and for inhibin-B the CVs were 15 and 18%. The limits of detection (LOD)s were FSH (0.05 IU/L), LH (0.05 IU/l), SHBG (0.23 nmol/l), T (0.23 nmol/l), E (18 pmol/l) and inhibin-B (3 pg/ml).

Table II Distribution of semen quality variables and reproductive hormones in the whole study group (n = 247).

	Mean	SD	Median	Percentile 05	Percentile 95
Testosterone (nmol/ml)	18.97	5.57	18.3	10.9	28.8
Estradiol (pmol/l)	74.7	21.4	73.0	44.0	113.0
SHBG (nmol/l)	25.5	9.84	24.0	12.0	43.0
LH (IU/l)	2.97	1.26	2.72	1.37	5.19
Inhibin-B (pg/ml)	181	60	174	90	287
FSH (IU/m)	2.48	1.40	2.21	0.89	5.59
Semen volume (ml)	3.24	1.52	3.00	1.20	5.70
Sperm concentration (10^6/ml)	66.1	58.7	49.0	4.38	175.0
Total sperm count (10^6)	194.8	167.3	141.4	12.2	507.3
Progressively motile (%)	57.8	15.6	60.5	26.3	78.0
Morphologically normal (%)	7.44	4.96	6.50	1.00	16.0
Total normal sperm count (10^6)	17.2	20.4	8.79	0.11	58.0

Limit of detection: FSH (0.05 IU/l), LH (0.05 IU/l), SHBG (0.23 nmol/l), T (0.23 nmol/l), E (18 pmol/l), inhibin-B (3 pg/ml).

Table III Identity of the targeted PFC compounds and corresponding LC-HRMS diagnostic signals, retention times and performances in term of limits of detection and limits of quantification.

Compound name	Abbreviation	Diagnostic signal HRMS (m/z)	Retention time	LOD (ng/ml)	LOQ (ng/ml)
Perfluoro-*n*-butanoic acid	PFBA	212.979	1.08	0.10	0.30
Perfluoro-*n*-[1,2,3,4-$^{13}C_4$] butanoic acid	**PFBA $^{13}C_4$**	**216.993**	**1.08**		
Perfluoro-*n*-pentanoic acid	PFPA	262.976	2.81	0.20	0.60
Perfluoro-*n*-hexanoic acid	PFHxA	312.973	5.22	0.10	0.30
Perfluoro-*n*-heptanoic acid	PFHpA	362.970	5.93	0.05	0.15
Perfluoro-*n*-octanoic acid	PFOA	412.966	6.39	0.05	0.15
perfluoro-*n*-[1,2,3,4-$^{13}C_4$]octanoic acid	**PFOA $^{13}C_4$**	**416.980**	**6.39**		
Perfluoro-*n*-nonanoic acid	PFNA	462.963	6.72	0.10	0.30
Perfluoro-*n*-[$^{13}C_9$]nonanoic acid	**PFNA $^{13}C_9$**	**471.993**	**6.72**		
Perfluoro-*n*-decanoic acid	PFDA	512.960	7.00	0.07	0.21
Perfluoro-*n*-[1,2-$^{13}C_2$]decanoic acid	**PFDA $^{13}C_2$**	**514.967**	**7.00**		
Perfluoro-*n*-undecanoic acid	PFUnA	562.957	7.22	0.50	1.5
Perfluoro-*n*-[1,2,3,4,5,6,7-$^{13}C_7$]undecanoic acid	**PFUnA $^{13}C_7$**	**569.980**	**7.22**		
Perfluoro-*n*-dodecanoic acid	PFDoA	612.954	7.42	0.20	0.60
Perfluoro-*n*-[1,2-$^{13}C_2$]dodecanoic acid	**PFDoA $^{13}C_2$**	**614.960**	**7.42**		
Potassium perfluoro-1-butanesulfonate	PFBS	298.943	4.09	0.10	0.30
Potassium perfluoro-1-hexanesulfonate	PFHxS	398.937	6.00	0.07	0.21
Sodium perfluoro-1-[$^{18}O_2$]hexanesulfonate	**PFHxS $^{18}O_2$**	**402.945**	**6.00**		
Potassium perfluoro-1-heptanesulfonate	PFHpS	448.933	6.39	0.07	0.21
Potassium perfluorooctanesulfonate	PFOS	498.930	6.72	0.01	0.03
Sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]octanesulfonate	**PFOS $^{13}C_4$**	**502.930**	**6.72**		
Sodium perfluoro-1-octanesulfonate acid	PFOSi	482.935	6.84	0.05	0.15
Sodium perfluoro-1-[1,2,3,4-$^{13}C_4$]-octanesulfonate	**PFOSi $^{13}C_4$**	**486.949**	**6.84**		

The names in bold are internal standards used for quantification.

We calculated FAI as (total T × 100/SHBG), and free testosterone (FT) from total T and SHBG assuming a fixed albumin level of 43.8 g/l as described by (Vermeulen *et al.*, 1999). Hormone ratios were calculated by simple division.

Semen analysis

Semen volume was assessed by weight. Sperm concentration was determined using a Bürker-Türk haemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Total sperm count (semen volume × sperm concentration) and percentage of progressively motile (WHO class A + B) and motile (WHO class A + B + C) spermatozoa were calculated (World Health Organization, 2010). Morphology slides were fixed and Papanicolaou stained, and assessed according to strict criteria (Menkveld *et al.*, 1990). Analysis of sperm concentration and motility was carried out in duplicates on fresh samples by one examiner per sample. Sperm morphology was assessed later in batches by two technicians. Semen analysis was performed in accordance with the WHO guidelines (World Health Organization, 2010) and has been described in detail previously (Jørgensen *et al.*, 2002). Motility was analysed within 60 min for 78% of samples, and 95% of samples were analysed within 90 min.

Statistics

Basic descriptive statistics were done for population characteristics, serum levels of reproductive hormones and PFCs, and semen parameters. Correlations between PFC concentrations, as well as possible confounders, were first explored using Spearman correlations (results not shown). Dependent variables were ln transformed (all reproductive hormone concentrations, ratios between hormones and semen volume), cubic root transformed (sperm concentration, total sperm count), squared (progressively motile) or square root transformed (morphologically normal) to achieve normality of distribution of residuals. Effects of the PFCs were modelled with each PFC as a continuous variable in linear regression models. PFC concentrations were ln transformed only when used as dependent variables (in analyses of temporal changes). *P*-values were not adjusted for multiple testing.

In linear regression models of associations between serum PFC levels and reproductive hormones, covariates were included by forward-selection, taking into account whether they were significant predictors of outcome and whether they changed the estimates by >10%. BMI was a significant predictor of outcome in models with T, E, SHBG, FAI, T/LH and T/E as dependent variables. Smoking (number of cigarettes smoked per day) was a significant predictor of outcome in models of T and FT. BMI and smoking were included as covariates in regression models for all reproductive hormones to facilitate interpretation of results.

In models of associations between serum PFC levels and semen variables, none of the tested covariates substantially changed the *P*-value or effect estimate. However, abstinence time was included in regression models for semen volume, concentration and total count, as abstinence time is accepted to be the most important confounder for these variables, and time from ejaculation to analysis was included in models of PFC levels and sperm motility. We also entered percentage total motile sperm (WHO A + B + C) instead of progressively motile sperm (WHO A + B), which gave essentially the same conclusions (results not shown). Percentage of morphologically normal sperm was left unadjusted, as no tested confounders were significant predictors of outcome or changed the effect estimate.

Other covariates were considered but not included in the final models: time of day of blood sample, ethnicity, units of alcohol consumed within the week prior to participation, *in utero* exposure to tobacco smoke, previous or current diseases (Table I), recent fever, recent use of medication and season. An interaction term between PFOS and cigarette smoking was statistically significant in the testosterone model only, suggesting that the more a subject smoked (cigarettes per day), the weaker the effect of PFOS on testosterone. Inclusion of this interaction term, however, did not change the effect estimate for the whole group and was thus also not included in the final models.

Differences between 2003 and 2008–2009 levels of PFOS, PFOA and PFNA were analysed in regression models correcting for smoking (cigarettes per day), alcohol intake and age, with year of examination as a fixed variable.

Data analysis was performed using PASW Statistics version18 (IBM, New York, USA).

Ethical approval

The study was conducted according to the Declaration of Helsinki, and was approved by the Danish National Committee on Biomedical Research Ethics (reference no. H-KF-289428). Participants gave written informed consent before participation.

Results

PFC levels

Among the 14 targeted PFC substances, 6 were detected in 99–100% of the analysed serum samples (Table IV). Regression analyses were done only for these six PFCs. PFHpA and PFUnA were detected in <50% of the analysed samples. All other monitored PFC compounds (PFBS, PFBA, PFPA, PFHxA, PFDoA and PFOSi) were detected in very few of the analysed serum samples (results not shown). Concentrations of the PFCs shown in Table IV were correlated with each other (all $P < 0.001$, Spearman's Rho 0.26–0.82). PFC levels were generally not correlated with age, BMI or date of participation within the years 2008–2009.

PFCs and reproductive hormones and semen quality

Table V shows the associations between serum PFCs and reproductive hormones and semen quality. PFOS showed negative associations with T, FT, FAI, T/LH, FT/LH and FAI/LH (all $P < 0.05$). PFOS was also negatively associated with estradiol, T/E ratio and inhibin-B/FSH ratio, and positively associated with SHBG, LH, FSH and inhibin-B, although this did not reach statistical significance. Entering quartiles of PFOS as a fixed variable in the regression models did not reveal signs of a specific threshold value for association with testosterone. When splitting participants into smokers and non-smokers, regression coefficients (95% CI) for effect of PFOS on testosterone were −0.005 (−0.022, 0.012) for smokers and −0.013 (−0.025, 0.00) for non-smokers, compared with the coefficient for the whole group of −0.010 (−0.020, 0.000) as shown in Table V. Figure 1 shows the estimates of serum concentrations of testosterone, FT and related hormone ratios for a man at the 5th and 95th percentiles of serum PFOS concentration (estimated from the linear regression models adjusted to a non-smoking man with the BMI of 23.0 kg/m^2): T is estimated to be 10% lower and FT is estimated to be 15% lower for a man with the highest (95th percentile) serum PFOS concentration compared with a man with the lowest PFOS level (5th percentile). Other PFCs were not significantly associated with reproductive hormones, but the regression coefficients were generally in the same direction, e.g. the estimates for effect on T were all negative.

Table IV Concentrations of PFCs determined in serum samples (n = 247).

PFC (ng/ml)	% >LOD	Mean ± SD	Percentiles				
			5th	25th	50th	75th	95th
PFHxS	100	0.81 ± 0.88	0.37	0.49	0.67	0.89	1.58
PFHpS	100	0.29 ± 0.13	0.15	0.20	0.26	0.33	0.52
PFOS	100	8.46 ± 3.74	4.28	6.23	7.79	9.81	14.59
PFOA	100	3.46 ± 1.99	1.82	2.58	3.02	3.86	6.15
PFNA	100	1.23 ± 0.63	0.64	0.88	1.07	1.41	2.41
PFDA	99[a]	0.38 ± 0.16	0.22	0.28	0.35	0.45	0.61

[a]Measurements below the LOD were assigned a value of LOD/$\sqrt{2}$.

PFHpS was negatively associated with percentage progressively motile sperm, but no other associations were found between serum PFC levels and any semen quality parameters. There were also no associations between testicular volume and serum PFC levels (results not shown).

Temporal trends in serum PFC

To compare the levels of PCF in the present study measured by LABERCA – ONIRIS (samples collected in 2008–2009) with previously published levels measured at the NERI, Aarhus University (samples collected in 2003), an inter-lab comparison was carried out using 25 of serum samples collected in 2003 and analysed in both laboratories. Simple scatterplots (Supplementary data) and Bland–Altman plots (not shown) exhibited good linear correlation between the two laboratories for PFOS, PFOA and PFNA but were somewhat less convincing for PFHxS and PFDA. However, absolute measured levels for all compounds were lower at LABERCA – ONIRIS than at NERI in the original analysis of samples collected in 2003. We adjusted for inter-laboratory differences between the two studies by multiplying the 'new' PFOS, PFOA and PFNA measurements by calculated factors of 1.41, 1.14 and 1.11, respectively, corresponding to the factors between the measurement levels of the two laboratories for these compounds.

Unadjusted values from both studies, as well as levels adjusted for inter-laboratory differences, are shown in Table VI. Serum PFOS and PFOA levels were lower in samples collected in 2008–2009 compared with 2003, while PFNA levels were higher (all $P < 0.002$ in regression analyses adjusted for confounders).

Discussion

Our results showed that higher serum PFOS concentrations were associated with lower serum levels of total and calculated free testosterone. These findings are consistent with some (Biegel *et al.*, 1995), but not all, animal studies (Butenhoff *et al.*, 2002). The effect estimates did not change significantly when correcting for relevant confounders, and confirmed the tendency of lower testosterone production that was suggested in our previous, smaller study of men participating in 2003. In the previous study, the regression coefficients from association models were analysed in the same way. There have been two other non-occupational studies of associations between PFC exposure and reproductive hormones in adult men (Raymer *et al.*, 2012; Specht *et al.*, 2012), neither of which demonstrated any associations between serum testosterone and PFC levels. Compared with Specht *et al.*, our group is a quite homogenous population with regard to age, BMI and other confounding factors. Raymer *et al.* found a positive correlation between serum LH and both PFOS and PFOA, but no potential confounders were considered in the analyses. Also, Raymer *et al.* investigated the associations in a heterogeneous group of men from an infertility clinic with a higher median LH than found in the present study, which would generally be consistent with a poorer Leydig cell function; it could be argued that the Raymer *et al.* study may include a proportion of men who have other important factors with a negative influence of Leydig cell function. These considerations may explain the discrepancy between published non-occupational studies and the results from the present study. However, effects of uncontrolled or residual confounding and chance findings due to multiple testing cannot be ruled out in this type of study. Other PFCs were detected in lower levels in serum than PFOS in this study and were not significantly associated with reproductive hormones.

Our inter-laboratory comparison of 25 samples showed good agreement for serum PFOS, PFOA and PFNA concentrations between the two laboratories. Both methods are valid, albeit the new analysis demonstrated a lower level. Following adjustment for this inter-laboratory variation, it seems reasonable to conclude that levels of serum PFOS and PFOA in young Danish men have indeed declined from 2003 to 2008–2009, and that the serum PFNA concentration has increased. Humans are exposed to a mixture of branched and linear forms of PFCs, including PFOS. One reason for the discrepancy in levels in the inter-laboratory comparison may be that the samples from 2003 were re-analysed using a method that distinguishes between linear and branched versions of some PFC congeners, which is not the case for all existing methods used to monitor these substances (Beesoon *et al.*, 2011; O'Brien *et al.*, 2011). Some of these results indicate a need for a more comprehensive and precise characterisation of the human exposure to PFCs, including the generation of more global contamination profiles (branched versus linear forms, main PFCs but also precursors and/or other representatives of this large class of substances). A more comprehensive assessment of the different sources of exposure and determinants of the measured internal doses are also required, as well as more toxicological studies dedicated to other PFC's representatives than PFOS and PFOA.

Table V Regression coefficients (95% CI) for change in transformed semen quality variables or reproductive hormones associated with a change in PFC of 1 ng/ml ($n = 247$).

	PFHxS	PFHpS	PFOS	PFOA	PFNA	PFDA
Semen variable (transformation)						
Volume (ln)[a]	0.051 (−0.017, 0.119)	0.321 (−0.162, 0.803)	0.015 (−0.001, 0.031)	−0.008 (−0.038, 0.022)	0.013 (−0.082, 0.108)	0.277 (−0.105, 0.659)
Concentration[a] (cubic Rt)	0.051 (−0.122, 0.224)	−0.086 (−1.234, 1.062)	0.009 (−0.031, 0.05)	0.029 (−0.047, 0.106)	0.105 (−0.139, 0.348)	0.218 (−0.755, 1.191)
Total count[a] (cubic Rt)	0.211 (−0.032, 0.453)	1.567 (−0.157, 3.291)	0.046 (−0.012, 0.103)	0.025 (−0.083, 0.133)	0.151 (−0.192, 0.495)	0.793 (−0.576, 2.162)
Progressively motile[b] (Sq)	−2.815 (−232, 227)	**−1761 (−3311, −212)**	−37.2 (−93.1, 18.7)	−4.426 (−109, 100)	−223 (−584, 138)	−1343 (−2759, 73.692)
Morphologically normal[c] (Sq Rt)	0.119 (−0.020, 0.257)	0.252 (−0.752, 1.257)	0.002 (−0.031, 0.035)	−0.012 (−0.074, 0.050)	−0.063 (−0.261, 0.134)	−0.097 (−0.884, 0.691)
Total normal count[a] (Cubic Rt)	0.155 (0.012, 0.298)	0.619 (−0.416, 1.653)	0.022 (−0.013, 0.056)	0.009 (−0.055, 0.074)	0.045 (−0.163, 0.254)	0.361 (−0.464, 1.185)
Hormone (transformation)						
Testosterone[d] (ln)	−0.012 (−0.054, 0.031)	−0.045 (−0.329, 0.239)	**−0.010 (−0.020, 0.000)**	−0.002 (−0.021, 0.017)	−0.059 (−0.118, 0.001)	−0.166 (−0.405, 0.072)
FAI[d] (ln)	0.015 (−0.041, 0.070)	−0.051 (−0.425, 0.323)	**−0.020 (−0.033, −0.006)**	0.011 (−0.014, 0.036)	−0.030 (−0.108, 0.049)	−0.307 (−0.621, 0.006)
FT[d] (ln)	−0.001 (−0.045, 0.043)	−0.057 (−0.353, 0.238)	**−0.016 (−0.026, −0.006)**	0.004 (−0.016, 0.023)	−0.052 (−0.114, 0.010)	−0.243 (−0.491, 0.005)
FT/LH[d] (ln)	0.008 (−0.056, 0.072)	−0.184 (−0.610, 0.243)	**−0.022 (−0.037, −0.007)**	−0.004 (−0.032, 0.024)	−0.051 (−0.140, 0.039)	−0.348 (−0.707, 0.010)
FAI/LH[d] (ln)	0.024 (−0.053, 0.101)	−0.165 (−0.681, 0.351)	**−0.025 (−0.043, −0.007)**	0.004 (−0.030, 0.038)	−0.024 (−0.133, 0.084)	−0.397 (−0.083, 0.036)
Testo/LH[d] (ln)	−0.002 (−0.062, 0.058)	−0.159 (−0.561, 0.243)	**−0.016 (−0.030, −0.002)**	−0.009 (−0.036, 0.018)	−0.053 (−0.137, 0.031)	−0.256 (−0.594, 0.082)
Testo/Estra[d] (ln)	−0.010 (−0.047, 0.026)	0.027 (−0.218, 0.273)	−0.003 (−0.011, 0.006)	−0.005 (−0.022, 0.011)	0.016 (−0.035, 0.068)	0.056 (−0.151, 0.263)
Estradiol[d] (ln)	−0.002 (−0.042, 0.038)	−0.072 (−0.341, 0.196)	−0.008 (−0.017, 0.002)	0.003 (−0.015, 0.021)	**−0.075 (−0.013, −0.019)**	−0.223 (−0.477, 0.002)
SHBG[d] (ln)	−0.026 (−0.082, 0.030)	0.006 (−0.370, 0.382)	0.009 (−0.004, 0.023)	−0.013 (−0.038, 0.012)	−0.029 (−0.108, 0.050)	0.141 (−0.176, 0.458)
LH[d] (ln)	−0.009 (−0.068, 0.049)	0.114 (−0.277, 0.505)	0.005 (−0.008, 0.019)	0.007 (−0.019, 0.033)	−0.005 (−0.088, 0.077)	0.090 (−0.240, 0.420)
FSH[d] (ln)	−0.022 (−0.098, 0.054)	0.090 (−0.419, 0.598)	0.007 (−0.012, 0.025)	0.024 (−0.009, 0.058)	0.085 (−0.021, 0.192)	0.421 (−0.005, 0.848)
Inhibin-B[d] (ln)	0.012 (−0.042, 0.066)	0.027 (−0.334, 0.388)	0.003 (−0.010, 0.016)	−0.006 (−0.030, 0.018)	−0.006 (−0.082, 0.070)	−0.057 (−0.361, 0.248)
Inhibin-B/FSH[d] (ln)	0.034 (−0.080, 0.147)	−0.063 (−0.826, 0.701)	−0.004 (−0.031, 0.024)	−0.031 (−0.081, 0.020)	−0.091 (−0.251, 0.068)	−0.478 (−1.119, 0.163)

[a]Adjusted for abstinence time.
[b]Adjusted for time to semen analysis.
[c]Unadjusted.
[d]Adjusted for BMI and smoking (cigarettes per day).
Statistically significant associations are highlighted in bold ($P < 0.05$).

Previously, we showed that men with high PFOS and PFOA levels combined had fewer morphologically normal sperm. This association was not seen in the present study, and the question stands whether the previous results could be a chance finding. However, the levels of both PFOA and PFOS have decreased between 2003 and 2008–2009, a decrease of >50% for PFOS. This meant that none of the men in the present study (2008–2009 participants) could be classified in the 'high PFC' category using the limits from the previous study of 2003 participants. This lack of highly exposed individuals in the current study could explain why we could not replicate our previous findings. A recent study in men from Greenland, Poland and Ukraine detected a reduction in the proportion of morphologically normal sperm in men with the highest exposure to PFOS (Toft *et al.*, 2012), corroborating our original findings. The temporal decrease in serum PFOS and PFOA levels is consistent with observations in other studies (Kato *et al.*, 2011) and a beneficial effect of the voluntary phase-out of production by the major manufacturer (3M Company) in 2002, and later regulatory measures (US Environmental Protection Agency 2012). PFOS is now included as a persistent organic pollutant in Annex B of the Stockholm Convention (2009).

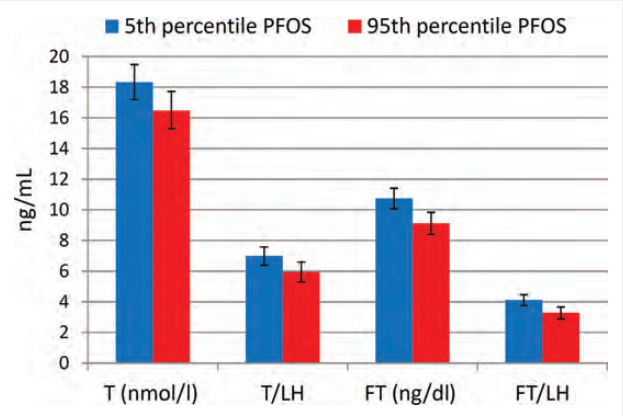

Figure 1 Estimated levels of testosterone (T), free testosterone (FT) and related hormone ratios for men with the lowest (blue) and highest (red) level of PFOS exposure. Bars are estimated T levels for a non-smoking man, BMI 23.0 kg/m^2, at the 5th (4.28 ng/ml) or 95th (14.6 ng/ml) percentile of PFOS exposure. Whiskers are confidence intervals for these estimated T levels from multivariate regression analyses).

In the present study, we found a negative association between serum PFHpS and sperm motility, but the results for other PFCs than PFHpS were not statistically significant. We have no biological explanation for this observation, as there are no data to support a specific effect of PFHpS on any aspect of spermatogenesis. Also, we found no background literature to suggest that sperm motility should be specifically affected by PFCs and so this may be a chance finding due to mass significance. On the other hand, there is very sparse knowledge of the mechanisms of action of PFCs, and especially PFHpS, and so we cannot exclude that this could be a real finding.

Our observation that testosterone, but not semen quality, was negatively associated with PFOS in this study with lower exposure levels than the previous study, may indicate that Leydig cells are more sensitive to PFC effects than spermatogenesis, or it may be that slight effects are simply easier to detect in this kind of study, because testosterone levels are less prone to inter-individual and inter-observer variation than semen quality measures.

PFOS is stable, with a half-life of years in humans (Olsen *et al.*, 2007), making a single serum measurement a good measure of internal exposure over enough time, so that it is reasonable to believe that effects on spermatogenesis and hormone regulation could take place. The reproductive hormone levels of these healthy young men were generally within the normal range for their age (Department of Growth and Reproduction, Copenhagen University Hospital, 2012), for men in both the highest and lowest PFOS exposure groups. A 10% lower T and 15% lower FT for men in the highest (95th percentile) exposure to PFOS compared with men in the 5th percentile of PFOS suggests that men living in areas of high environmental contamination may, however, still have a slightly increased risk of adverse reproductive effects.

Table VI Temporal trends in serum concentrations of PFCs.

PFC ng/ml	Mean ± SD	Selected percentiles				
		5th	25th	50th	75th	95th
PFOS 2008–2009 raw	8.46 ± 3.74	4.28	6.23	7.79	9.81	14.59
PFOS 2008–2009 adjusted[a,b]	11.9 ± 5.26	6.02	8.77	11.0	13.8	20.5
PFOS 2003	25.3 ± 7.85	14.3	19.7	24.5	28.7	39.6
PFOA 2008–2009 raw	3.46 ± 1.99	1.83	2.58	3.02	3.86	6.15
PFOA 2008–2009 adjusted[a,b]	3.97 ± 2.28	2.09	2.95	3.47	4.42	7.05
PFOA 2003	4.80 ± 1.33	2.77	3.72	4.85	5.68	6.88
PFNA 2008–2009 raw	1.23 ± 0.63	0.64	0.88	1.07	1.41	2.41
PFNA 2008–2009 adjusted[a,b]	1.35 ± 0.70	0.70	0.97	1.17	1.56	2.66
PFNA 2003	0.89 ± 0.45	0.43	0.59	0.80	1.02	1.61

[a]Adjusted for inter-laboratory difference to allow for comparison with 2003 levels.
[b]Difference between samples collected in 2003 ($n = 105$) and 2008–2009 ($n = 247$) was statistically significant (all $P < 0.002$) after adjustment for smoking, alcohol intake and age.

On the basis of the few studies available on this topic, it is usually accepted that PFOA and PFOS are not metabolized in humans, and are mainly distributed to the liver, kidneys and serum (reviewed in Loccisano *et al*., 2011). It is established that PFOS and PFOA have an affinity for proteins and can bind to albumin, an abundant protein in the plasma with a wide range of ligands. It is estimated that 90% of PFOA in serum is bound to albumin (Han *et al*., 2003). Animal studies have suggested a decrease in thyroid hormone (T_4) after exposure to PFCs, which may be mediated by competitive binding of PFOS to thyroid hormone transport proteins, rather than changes in thyroid hormone synthesis or signalling (Weiss *et al*., 2009). PFOS can also bind to SHBG, although with low affinity, and can displace estradiol and testosterone at very high concentrations (Jones *et al*., 2003). Thus, it is possible that high concentrations of PFOS would decrease the amount of SHBG available for testosterone binding without decreasing the total amount of SHBG in serum. This could then lead to an under-estimation of calculated free testosterone using the total SHBG levels that we measure in serum. Alternative mechanisms of action for PFOS on testosterone include the inhibition of steroidogenic enzyme activity (Zhao *et al*., 2010), weak estrogenic activity (Benninghoff *et al*., 2011) or changes in cell membrane fluidity and permeability (Hu *et al*., 2003).

Several classes of environmental endocrine disrupting chemicals have anti-androgenic effects, mediated by mechanisms such as interference with the androgen receptor, androgen production or metabolism, or signalling in the hypothalamic–pituitary–gonadal axis, which underlines the importance of considering mixture effects of chemicals with similar outcomes (Kortenkamp and Faust, 2010; Søeborg *et al*., 2012). Tolerable daily intakes (TDI) have been set for PFOS and PFOA (European Food Safety Authority, 2012), and most population-based studies indicate exposure to PFOS and PFOA below these. However, the TDIs are based on liver damage outcome and are not anti-androgenic effects, so currently we have no way to assess relevant cumulated risk for the PFCs measured in this study. Combination effects with exposure to other compounds with similar effects may, for highly exposed or highly sensitive individuals, contribute to the development of a hypogonadal state with lower testosterone production and an increased risk of obesity, decreased muscle mass, osteoporosis and type II diabetes.

In conclusion, we observed that serum testosterone was slightly, but statistically significantly, lower in men with higher serum PFOS, consistent with the tendency observed in a previously published smaller study in a separate sample of men from the same segment of the population. Our previously observation of fewer morphologically normal sperm in the group with the highest combined PFOS and PFOA exposure was, however, not confirmed, possibly due to a significant decrease in serum levels of PFOA and PFOS since our first study. The temporal changes in exposure levels indicate that recent regulatory measures are effective, and although the effects estimate of PFOS on testosterone levels is modest in this population, our results suggest that men living in areas of high environmental PFOS contamination may still be at risk of adverse reproductive effects.

Supplementary data

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

Authors' roles

U.N.J., J.P.A., N.E.S., A.M.A. and N.J. participated in the study design. U.N.J., B.V., J.P.A., M.B.J., P.M., A.M.A., B.L.B. and N.J. participated in the study execution and acquisition of data. All authors participated in the data analysis, manuscript drafting and critical discussion and gave final approval of the submitted manuscript.

Funding

The authors received financial support from the European Commission (DEER, FP7-2007-212844), the Danish Agency for Science, Technology and Innovation (grant nos. 27107068 and 09-067180), Rigshospitalet (grant no. 961506336), the University of Copenhagen, the Danish Ministry of Health and the Danish Environmental Protection Agency (MST-621-00013) and the Kirsten and Freddy Johansen Foundation (grant no. 95-103-72087). The funding organizations played no role in the design and conduct of the study, in the collection, management, analysis and interpretation of the data, or in the presentation, review or approval of the manuscript.

Conflict of interest

None declared.

References

Beesoon S, Webster GM, Shoeib M, Harner T, Benskin JP, Martin JW. Isomer profiles of perfluorochemicals in matched maternal, cord, and house dust samples: manufacturing sources and transplacental transfer. *Environ Health Perspect* 2011;**119**:1659–1664.

Begley TH, Hsu W, Noonan G, Diachenko G. Migration of fluorochemical paper additives from food-contact paper into foods and food simulants. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2008; **25**:384–390.

Benninghoff AD, Bisson WH, Koch DC, Ehresman DJ, Kolluri SK, Williams DE. Estrogen-like activity of perfluoroalkyl acids in vivo and interaction with human and rainbow trout estrogen receptors in vitro. *Toxicol Sci* 2011;**120**:42–58.

Biegel LB, Liu RC, Hurtt ME, Cook JC. Effects of ammonium perfluorooctanoate on Leydig cell function: in vitro, in vivo, and ex vivo studies. *Toxicol Appl Pharmacol* 1995;**134**:18–25.

Butenhoff J, Costa G, Elcombe C, Farrar D, Hansen K, Iwai H, Jung R, Kennedy G Jr, Lieder P, Olsen G *et al*. Toxicity of ammonium perfluorooctanoate in male cynomolgus monkeys after oral dosing for 6 months. *Toxicol Sci* 2002;**69**:244–257.

Cook JC, Murray SM, Frame SR, Hurtt ME. Induction of Leydig cell adenomas by ammonium perfluorooctanoate: a possible endocrine-related mechanism. *Toxicol Appl Pharmacol* 1992;**113**:209–217.

Department of Growth and Reproduction, Copenhagen University Hospital, 2012 Normal ranges for reproductive hormones in Danish men [Danish]. http://www.rigshospitalet.dk/NR/rdonlyres/BF045510-9714-4A44-9906-FD5B02E5CD13/0/Referencem%c3%a6nd.pdf. (8 December 2012, date last accessed).

European Food Safety Authority. Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts. Scientific opinion of the panel on contaminants in the food chain, 2012. http://www.efsa.europa.eu/en/efsajournal/doc/653.pdf. (8 December 2012, date last accessed).

Gilliland FD. *Fluorocarbons and Human Health: Studies in an Occupational Cohort*. Minneapolis, MN: University of Minnesota, 1992.

Han X, Snow TA, Kemper RA, Jepson GW. Binding of perfluorooctanoic acid to rat and human plasma proteins. *Chem Res Toxicol* 2003;**16**:775–781.

Hu W, Jones PD, Decoen W, King L, Fraker P, Newsted J, Giesy JP. Alterations in cell membrane properties caused by perfluorinated compounds. *Comp Biochem Physiol C Toxicol Pharmacol* 2003;**135**:77–88.

Joensen UN, Bossi R, Leffers H, Jensen AA, Skakkebæk NE, Jørgensen N. Do perfluoroalkyl compounds impair human semen quality? *Environ Health Perspect* 2009;**117**:923–927.

Jones PD, Hu W, De CW, Newsted JL, Giesy JP. Binding of perfluorinated fatty acids to serum proteins. *Environ Toxicol Chem* 2003;**22**:2639–2649.

Jørgensen N, Carlsen E, Nermoen I, Punab M, Suominen J, Andersen AG, Andersson AM, Haugen TB, Horte A, Jensen TK *et al*. East-West gradient in semen quality in the Nordic-Baltic area: a study of men from the general population in Denmark, Norway, Estonia and Finland. *Hum Reprod* 2002;**17**:2199–2208.

Kannan K, Corsolini S, Falandysz J, Fillmann G, Kumar KS, Loganathan BG, Mohd MA, Olivero J, Van Wouwe N, Yang JH *et al*. Perfluorooctanesulfonate and related fluorochemicals in human blood from several countries. *Environ Sci Technol* 2004;**38**:4489–4495.

Kato K, Wong LY, Jia LT, Kuklenyik Z, Calafat AM. Trends in exposure to polyfluoroalkyl chemicals in the U.S. Population: 1999–2008. *Environ Sci Technol* 2011;**45**:8037–8045.

Kissa E. *Fluorinated Surfactants and Repellents*, 2nd edn. Surfactant Science Series, Vol. **97**. CRC Press, 2001.

Kortenkamp A, Faust M. Combined exposures to anti-androgenic chemicals: steps towards cumulative risk assessment. *Int J Androl* 2010; **33**:463–474.

Loccisano AE, Campbell JL Jr, Andersen ME, Clewell HJ III. Evaluation and prediction of pharmacokinetics of PFOA and PFOS in the monkey and human using a PBPK model. *Regul Toxicol Pharmacol* 2011;**59**:157–175.

Menkveld R, Stander FS, Kotze TJ, Kruger TF, van Zyl JA. The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum Reprod* 1990;**5**:586–592.

O'Brien JM, Kennedy SW, Chu S, Letcher RJ. Isomer-specific accumulation of perfluorooctane sulfonate in the liver of chicken embryos exposed in ovo to a technical mixture. *Environ Toxicol Chem* 2011;**30**:226–231.

Olsen GW, Gilliland FD, Burlew MM, Burris JM, Mandel JS, Mandel JH. An epidemiologic investigation of reproductive hormones in men with occupational exposure to perfluorooctanoic acid. *J Occup Environ Med* 1998;**40**:614–622.

Olsen GW, Burris JM, Ehresman DJ, Froehlich JW, Seacat AM, Butenhoff JL, Zobel LR. Half-life of serum elimination of perfluorooctanesulfonate,perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. *Environ Health Perspect* 2007;**115**:1298–1305.

Ravnborg TL, Jensen TK, Andersson AM, Toppari J, Skakkebæk NE, Jørgensen N. Prenatal and adult exposures to smoking are associated with adverse effects on reproductive hormones, semen quality, final height and body mass index. *Hum Reprod* 2011;**26**:1000–1011.

Raymer JH, Michael LC, Studabaker WB, Olsen GW, Sloan CS, Wilcosky T, Walmer DK. Concentrations of perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA) and their associations with human semen quality measurements. *Reprod Toxicol* 2012;**33**:419–427.

Shi Z, Zhang H, Liu Y, Xu M, Dai J. Alterations in gene expression and testosterone synthesis in the testes of male rats exposed to perfluorododecanoic acid. *Toxicol Sci* 2007;**98**:206–215.

Søeborg T, Frederiksen H, Andersson AM. Cumulative risk assessment of phthalate exposure of Danish children and adolescents using the hazard index approach. *Int J Androl* 2012;**35**:245–252.

Specht IO, Hougaard KS, Spano M, Bizzaro D, Manicardi GC, Lindh CH, Toft G, Jonsson BA, Giwercman A, Bonde JP. Sperm DNA integrity in relation to exposure to environmental perfluoroalkyl substances—a study of spouses of pregnant women in three geographical regions. *Reprod Toxicol* 2012;**33**:577–583.

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

Stockholm Convention, 2009 http://chm.pops.int/Convention/ThePOPs/ListingofPOPs/tabid/2509/Default.aspx. (8 December 2012, date last accessed).

Toft G, Jonsson BA, Lindh CH, Giwercman A, Spano M, Heederik D, Lenters V, Vermeulen R, Rylander L, Pedersen HS *et al*. Exposure to perfluorinated compounds and human semen quality in arctic and European populations. *Hum Reprod* 2012;**27**:2532–2540.

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.

Vermeulen A, Verdonck L, Kaufman JM. A critical evaluation of simple methods for the estimation of free testosterone in serum. *J Clin Endocrinol Metab* 1999;**84**:3666–3672.

Weiss JM, Andersson PL, Lamoree MH, Leonards PE, van Leeuwen SP, Hamers T. Competitive binding of poly- and perfluorinated compounds to the thyroid hormone transport protein transthyretin. *Toxicol Sci* 2009;**109**:206–216.

White SS, Fenton SE, Hines EP. Endocrine disrupting properties of perfluorooctanoic acid. *J Steroid Biochem Mol Biol* 2011;**127**:16–26.

World Health Organization. *WHO Laboratory Manual for the Examination and Processing Of Human Semen,* 5th edn, 2010. http://whqlibdoc.who.int/publications/2010/9789241547789_eng.pdf. (8 December 2012, date last accessed).

Zhao B, Hu GX, Chu Y, Jin X, Gong S, Akingbemi BT, Zhang Z, Zirkin BR, Ge RS. Inhibition of human and rat 3beta-hydroxysteroid dehydrogenase and 17beta-hydroxysteroid dehydrogenase 3 activities by perfluoroalkylated substances. *Chem Biol Interact* 2010; **188**:38–43.