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Exposure to perfluorinated compounds and human semen quality in arctic and European populations

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BACKGROUND: Perfluorinated compounds (PFCs) have been suspected to adversely affect human reproductive health. The aim of this study was to investigate the associations between PFC exposure and male semen quality.

METHODS: PFCs were measured in serum from 588 partners of pregnant women from Greenland, Poland and Ukraine who provided a semen sample, using liquid chromatography tandem mass spectrometry. Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorohexane sulfonic acid (PFHxS) and perfluorononanoic acid (PFNA) could be detected in >97% of the samples. The associations between levels of these compounds and semen volume, sperm concentration, total sperm count, motility and morphology were assessed.

RESULTS: Across countries, sperm concentration, total sperm count and semen volume were not consistently associated with PFOS, PFOA, PFHxS or PFNA levels. The proportion of morphologically normal cells was 35% lower [95% confidence interval (CI): 4–66%) for the third tertile of PFOS exposure as compared with the first. A similar reduction was found in relation to increasing PFHxS levels. At the third PFOA exposure tertile, the percentage of motile spermatozoa was 19% (95% CI: 1 to 39%) higher than in the first.

CONCLUSIONS: The most robust finding in the present study was the negative associations between PFOS exposure and sperm morphology suggesting adverse effects of PFOS on semen quality, possibly due to interference with the endocrine activity or sperm membrane function. It cannot be excluded that this association and the positive association between PFOA and semen motility, which was not consistent across countries, might represent a chance finding due to the multiple statistical tests being performed.

Key words: PFOS / PFOA / perflourinated compounds / semen quality / sperm morphology

Introduction

Since the middle of the last century, the annual production of perfluorinated compounds (PFCs), including perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), has been in the range of several hundred metric tons per year (Lau *et al.*, 2004). PFCs are used in a large variety of applications especially in surface coatings, making products water and oil resistant. Several of the compounds are approved for use in food packing materials, food containers and cooking pans and may thus be transferred to food (Renner, 2001). PFCs are highly biopersistent with a half-life in humans of ~5 years (Olsen *et al.*, 2007). PFOS has been added to Annex B of the Stockholm Convention on Persistent Organochlorine Pollutants in 2009 and the production and use of PFOS has been regulated in Europe since 2008 (European Commission 2006, 1998). However, modeling of exposure levels of PFOS in the environment indicates only slow decay in temperate regions and even an increase can be expected in Arctic regions until 2030 (Armitage *et al.*, 2009). PFCs have been detected

in human populations from all over the world, but with considerable variation among populations as far as their level of exposure is concerned. The highest blood concentration is found among workers in PFC-producing plants, with the mean PFOS concentrations of 1000–2000 ng/ml and PFOA concentrations of ~5000 ng/ml, whereas general populations on average have concentrations of ~35 ng/ml PFOS and 5 ng/ml PFOA (Lau *et al.*, 2004).

Male reproductive toxicity of PFCs has mainly been evaluated in animal models, where PFOS and PFOA were shown to reduce testosterone production in mice or rats (Biegel *et al.*, 1995; Wan *et al.*, 2011) and PFOS was associated with reduced epididymal sperm counts in mice (Wan *et al.*, 2011). As far as human studies are concerned, a study among human workers at a PFOA-producing facility found no association between PFOA exposure and reproductive hormones (Olsen *et al.*, 1998), but a recent study among 105 young Danish men showed an association between combined PFOS and PFOA exposure and the proportion of morphologically normal sperm cells, and suggested associations with several other semen parameters and levels of reproductive hormones, although these were not statistically significant (Joensen *et al.*, 2009). However, another recent study evaluated the semen quality among 256 infertility patients in relation to PFOS and PFOA in serum and semen and found no association between PFOS or PFOA levels and sperm concentration, volume or motility (Raymer *et al.*, 2011).

Thus, it is not possible to draw firm conclusions regarding possible impacts of PFC exposure on human semen quality based on the available data. The aim of the present study was, therefore, to further evaluate the possible association between PFC exposure and biomarkers of male reproductive health in a larger population of almost 600 men from Greenland, Poland and Ukraine, representing individuals with considerable variation in PFC exposure (Lindh *et al.*, 2012), allowing us to evaluate the potential effects across a range of exposure levels that can be expected in non-occupationally exposed men.

Materials and Methods

Study populations

The present study is part of a European study on fertility (www.inuendo.dk/clear) using a uniform protocol for data collection in Greenland, Kharkiv in Ukraine and Warsaw in Poland (Toft *et al.*, 2005a).

The population for this study is based on the previously established INUENDO cohort, including in total 798 men and 2269 pregnant women (Toft *et al.*, 2005a). To achieve a uniform study population, we excluded the fishermen population from Sweden, where the males were not partners of pregnant women, and the remaining cohort consisted of 607 male partners of 1710 pregnant women. In the present study we included 588 (97%) of these males who provided a semen sample, filled in a questionnaire on lifestyle, occupation and medical history and provided a blood sample which could be retrieved from a biobank and analysed for PFCs in 2011. All included men were 18 years or older at the time of enrolment. The participation rates in the present study varied from 29 in Warsaw and 36 in Kharkiv to 79% in Greenland. Study populations and data collection procedures have been described in detail elsewhere (Toft *et al.*, 2005a).

Semen samples

All semen samples were collected by masturbation. The subject was asked to abstain from sexual activities for at least 2 days before collecting the sample, and to report the actual abstinence time. The sample was kept close to the body to maintain a temperature close to 37°C if transport to the local hospital after collection was necessary. Analysis of semen samples were initiated within 1 h after ejaculation for 83% of the samples. The samples were analysed for concentration, motility and morphology according to a manual for the project based on the World Health Organization (WHO, 2009) manual for basic semen analysis. Briefly, the sperm concentration was determined in duplicate using an improved Neubauer Hemacytometer. The sperm motility was determined by counting the proportion of (i) rapid progressive spermatozoa; (ii) slow progressive spermatozoa; (iii) non-progressive motile spermatozoa and (iv) immotile spermatozoa, among 100 spermatozoa within each of two fresh drops of semen.

Semen samples from Warsaw and Kharkiv were analysed for concentration and motility at one central hospital in each region, whereas the samples from Greenland were analysed at the local hospitals or nursing stations scattered all over the country. One person performed all the semen analysis in each of the three countries. These three persons were previously trained in a quality control program set up specifically for this study (Toft *et al.*, 2005b). The quality control program ensured good agreement between observers during the data collection in the assessment of sperm concentration and sperm motility with a coefficient of variation (CV) of 8.1 and 11%, respectively. Sperm cells from all populations were stained and analyzed centrally at the Skåne University Hospital in Malmö, Sweden. Abnormalities were classified as head defects, midpiece defects, tail defects, cytoplasm drop and immature spermatozoa. The morphology was evaluated for at least 200 sperm in each sample by two technicians taking part in the NAFA-ESHRE (Nordic Association for Andrology, European Society of Human Reproduction and Embryology) external quality control program.

Collection of blood samples

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, Gothenburg, Sweden). A piece of aluminum foil was placed on top of the bottles which were then sealed. Sera were stored at −20°C until shipment, but refrigeration was accepted for up to 4 days. After the arrival at the analyzing laboratory, the samples were stored at −80°C until analyses.

Analysis of PFCs

The analysis of PFOS, PFOA, PFHxS and PFNA was performed by liquid chromatography tandem mass spectrometry (LC/MS/MS). Aliquots of 100 μl serum were added with labeled internal standards for all evaluated compounds and the proteins were precipitated with acetonitrile and vigorously shaking for 30 min. The samples were thereafter centrifuged and analyzed using an LC (UFLC^{XR}, SHIMADZU Corporation, Kyoto, Japan) connected to a hybrid triple quadrupole linear ion trap mass spectrometer equipped with a TurboIonSpray source (QTRAP 5500, Applied Biosystems). The MS analyses were carried out using selected reaction monitoring in the negative ion mode. The reproducibility of the method determined from duplicate samples analyzed at different days is described in detail elsewhere (Lindh *et al.*, 2012). Limits of detection (LOD), determined as the concentrations corresponding to three times the standard deviation of the responses in chemical blanks, were the following: PFOS 0.2 ng/ml; PFOA 0.6 ng/ml; PFHxS 0.06 ng/ml and PFNA 0.2 ng/ml.

The analyses of PFOS and PFOA are part of the Round Robin intercomparison program (Professor Dr. med. Hans Drexler, Institute and Out-Patient Clinic for Occupational, Social and Environmental Medicine,

University of Erlangen-Nuremberg, Germany) with results within the tolerance limits. The samples were analysed in triplicates and the results reported are the average of the two closest measurements from the samples worked-up and analyzed on different days. Samples below LOD were included in the analyses as the value reported from the mass spectrometer.

Statistical analysis

We analyzed exposure–response relations within each of the three populations and since population did not appear to be a strong effect modifier, we also performed analyses across the three populations. The individual populations were divided into tertiles of exposure based on measured exposure within each country, and in the analysis of the combined effects across populations, tertiles were made based on the exposure distribution in the combined population. First, we evaluated the difference between the tertiles and presented the results as the crude medians in the three tertiles and the calculated percent difference from the first tertile to the second and third tertile adjusted for the below mentioned potential confounders, using a general linear regression model. Secondly, we evaluated trends of exposure outcome associations by a second set of general linear regression models, with the exposure included as a continuous variable.

Due to the non-normal distributed exposures, we transformed all exposure variables by taking the natural logarithm when included in the model as a continuous variable. To improve normality and homogeneity of variance of the residuals in the tested associations, sperm concentration, total sperm count, volume, percentage motile sperm cells and percentage of morphologically normal sperm were ln-transformed as well. Model fit was evaluated by visual inspection of residual versus predicted values. Since cubic root transformations may more optimally transform sperm concentration and total sperm counts (Handelsman, 2002), and ln-transformations may not markedly improve normality for sperm motility and normal sperm cell morphology, models with the cubic root-transformed sperm concentration and total sperm count and untransformed sperm motility and normal sperm cell morphology were compared with ln-transformed models. Since results from the ln-transformed models did not deviate from the alternative models, the results from the ln-transformed models were presented to ease the interpretation of the results. All semen outcomes were, according to an *a priori* decision, adjusted for potential confounding effects of known determinants of semen quantity or quality including abstinence time and age of the subject (both logarithmic transformed); spillage (yes/no); current smoking (yes/no); ever urogenital infections (yes/no) and body mass index (kg/m^2; BMI; <20; 20–25 and >25) (Ramlau-Hansen *et al.*, 2007; Ochsendorf, 2008; Stewart *et al.*, 2009; Molina *et al.*, 2010). Univariate associations of covariates and sperm concentration is presented in Supplementary data, Table SI. The analysis of sperm motility was additionally restricted to samples analyzed within 1 h after collection, and the volume and total sperm count were restricted to samples from individuals reporting no spillage. In combined analyses, across populations represented as different countries, country was included as a potential confounder.

Since the measured PFCs are highly correlated, mutual adjustments for other exposures would probably overadjust the observed associations and mutual adjustments are therefore only presented as subanalyses.

Statistical analyses were performed using the SAS statistical software version 9.1 (SAS Institute, Inc., Cary, NC, USA).

To further study the shape of the exposure–response relations, we used a generalized additive model (GAM) to allow for possible non-linear relations between (ln-transformed) exposure variables and outcomes, adjusting for the same confounders as in the linear model. Exposure–response relations were performed both within and across the three populations. Degree of smoothing was selected by generalized cross-validation as implemented in the R package mgcv (Wood, 2011).

Results

Characteristics of the study population

In total, 588 persons delivered a semen sample and had a blood sample analyzed for PFCs and were therefore included in the study. PFOS and PFNA could be detected in all these samples, whereas PFHxS was below the detection limit in 1 sample and PFOA was below the detection limit in 16 samples corresponding to 3% of the samples.

Table I shows the distribution of PFC exposure between countries and combined, including tertile borders used in subsequent analyses. The highest levels of exposure to PFOS, PFHxS and PFNA were found in Greenland, whereas the median PFOA was marginally higher in Poland, and for all exposures the lowest levels were found in Ukraine. Seafood consumption, area of living and age were the main determinants of the high PFOS level in Greenland. A detailed description of differences in exposure between populations and determinants of PFC exposure can be found in Lindh *et al.* (2012).

The characteristics of the study populations are presented in Table II. Marked differences are observed in abstinence time, reported spillage, urogenital infections and smoking prevalence between countries, but semen outcomes are rather similar. From Supplementary data, Table SII, it can be observed that covariate distribution differs between exposure groups across countries, probably primarily due to differences between countries in exposure and covariate distribution.

PFOS and semen quality

The associations between PFOS exposure and semen quality are presented in Table III.

The semen volume and the proportion of motile sperm were not associated with PFOS exposure in any of the populations or in the combined estimate in analysis between tertiles of exposure. However, a few significant associations with sperm concentration and total sperm count were found in the analyses based on single countries including an estimated 53% higher sperm concentration [95% confidence interval (CI): 12; 93%] and a 57% (95% CI: 11; 103%) higher total sperm count in the second tertile of PFOS exposure compared with the first in the Polish population. No significant associations between PFOS exposure and sperm concentration or total sperm count were found in the other populations or in the combined estimate. Furthermore, when analyzed in relation to continuous PFOS exposure, we found no significant association with the semen volume, motility, concentration or total sperm count.

In the combined estimate, the proportions of normal cells were 22% (95% CI: 1; 44%) and 35% (95% CI: 4; 66%) lower at the second and third tertiles of PFOS exposure compared with the first, respectively. Multiple regression analysis on continuous PFOS exposure suggested a dose–response association ($P = 0.06$) on the combined estimate across countries and within Poland ($P = 0.10$) and Ukraine ($P = 0.08$), but PFOS was not associated with the proportion of normal sperm cells in Greenland ($P = 0.82$).

This is further supported by spline analysis indicating an overall downward trend in percent normal sperm in all countries, but not as strongly in Greenland as in the other populations (Supplementary data, Fig. S1).

Table I Median PFC serum concentrations and tertile borders in men from Greenland, Poland and Ukraine.

	Greenland (n = 196)			Poland (n = 189)			Ukraine (n = 203)			All (n = 588)		
PFC	Median	P33[a]	P66[b]	Median	P33[a]	P66[b]	Median	P33[a]	P66[b]	Median	P33[a]	P66[b]
PFOS (ng/ml)	44.7	38.8	56.1	18.5	15.4	21.2	7.6	6.0	8.5	18.4	11.9	27.3
PFOA(ng/ml)	4.5	4.2	5.2	4.8	4.2	5.6	1.3	1.0	1.6	3.8	2.5	4.7
PFHxS(ng/ml)	2.2	1.9	2.7	1.2	1.0	1.3	0.3	0.3	0.4	1.1	0.7	1.5
PFNA(ng/ml)	1.7	1.3	2.4	1.2	1.0	1.3	1.0	0.8	1.2	1.2	1.0	1.5

[a]P33 = 33rd percentile = upper level of the first tertile.
[b]P66 = 66th percentile = upper level of the second tertile.

Table II Characteristics and semen outcomes of the included study populations presented as the median and 5th–95th percentiles for continuous variables and n (%) for dichotomous variables.

Semen outcomes and covariates[a]	Greenland	Poland	Ukraine
Continuous variables (median, 5th–95th percentiles)			
Sperm concentration (10^6/ml)	52.6 (11.0–199.0)	64.0 (7.2–257.5)	58.9 (10.2–175.5)
Sperm volume (ml)	3.2 (1.1–6.8)	3.5 (1.1–7.2)	3.1 (1.3–7.3)
Total sperm count (10^6)	184.4 (27.4–692.3)	197.2 (18.7–1071.2)	175.0 (24.4–651.3)
Percent motile sperm (A + B)	60 (21.0–81.0)	63.5 (15.0–86.0)	55.5 (10.0–87.0)
Percent normal sperm	6.0 (2.0–13.0)	6.0 (2.0–14.0)	7.0 (1.0–15.0)
Age (years)	31.3 (20.8–43.2)	29.6 (25.3–36.8)	26.2 (20.7–39.6)
Abstinence time (days)	3.0 (0.5–7.0)	4.0 (1.0–30.0)	3.0 (1.5–7.0)
Body mass index (kg/m^2)	25.5 (20.5–31.1)	25.4 (20.5–31.8)	24.0 (19.6–29.5)
Dichotomous covariates (n (%)			
Reported spillage	21 (11)	10 (5.3)	34 (16.8)
Previous urogenital infections	162 (83)	10 (5.3)	10 (5.0)
Current smoking	141 (72)	53 (28)	134 (67)

[a]The following number of observations were available for the included outcomes and covariates: sperm concentration; sperm volume and total sperm count, n = 588; percent motile sperm, n = 580; percent normal sperm, n = 586; age, n = 584; abstinence time, n = 542; body mass index, n = 581; reported spillage (yes/no) n = 588; urogenital infections, n = 583; smoking, n = 584.

In order to identify any specific effects of PFOS exposure on sperm morphology, we analyzed PFOS exposure in relation to head defects, midpiece defects, tail defects, cytoplasm drop and immature spermatozoa. Results of the combined estimates across countries are presented in Table IV indicating a 28% (95% CI 7; 50%) increased proportion of tail defects in the second as compared with the first tertile of PFOS exposure. In general, all measured aspects of sperm morphology were slightly higher at the second and third tertiles of exposure compared with the first, although this was not statistically significant. No significant associations were found in relation to continuous PFOS exposure for the specific sperm morphology outcomes. In country-specific analyses, we did not observe significant associations between PFOS exposure and specific morphological effects (data not shown).

PFOA and semen quality

Regarding PFOA exposure, Table V shows no associations between PFOA and sperm concentration, total sperm count, semen volume or percent normal sperm within or across countries both in analyses based on tertiles of exposure and based on continuous exposure.

The proportion of motile sperm was 36% (CI: 6; 45%) higher at the third PFOA tertile in Greenland and positively associated with PFOA in the multiple regression analysis with PFOA as a continuous measure in Greenland ($P < 0.01$), but not in the other countries. The combined analysis across countries indicated a 19% (CI: 1; 39%) higher sperm motility in the third tertile of exposure, which was also reflected in the multiple regression analysis ($P < 0.01$). Spline regressions supported these findings with markedly stronger associations in Greenland compared with the other populations (Supplementary data, Fig. S2).

PFHxS and PFNA and semen quality

PFHxS and PFNA were not associated with sperm concentration, volume, total count or percent motile sperm in analyses based on tertiles of exposure or continuous exposure (Table VI). However, for PFHxS a 35% (95% CI: 1; 70%) lower proportion of normal sperm were found at the highest tertile compared with the first, and a

Table III Semen quality in relation to PFOS exposure in tertiles and continuous trend tests.

Parameter	First tertile		Second tertile		Third tertile		Adjusted difference from first tertile (95% CI)[a]		Trend test	
	n	Median (P25; P75)	*n*	Median (P 25; P 75)	*n*	Median (P 25; P 75)	Second tertile	Third tertile	*P* crude[b]	*P* adjusted[c]
Sperm concentration (10^6/ml)										
Greenland	65	44 (26; 84)	65	53 (38; 91)	66	62 (3; 106)	0 (−29; 29)	12 (−18; 42)	0.07	0.14
Poland	63	54 (18; 98)	63	75 (39; 127)	63	72 (30; 117)	53 (12; 93)*	25 (−14; 64)	0.29	0.41
Ukraine	67	59 (35; 97)	68	63 (33; 102)	68	53 (32; 91)	9 (−20; 38)	−15 (−44; 15)	0.71	0.40
Combined	196	61 (33; 97)	195	58 (31; 114)	197	58 (30; 98)	14 (−15; 43)	22 (−21; 64)	0.79	0.51
Volume (ml)[d]										
Greenland	59	3.3 (2.2; 5.0)	58	3.5 (2.6; 4.8)	58	3.2 (2.5; 4.1)	−5 (−23; 14)	−7 (−26; 13)	0.51	0.27
Poland	58	3.4 (2.4; 4.9)	61	3.6 (2.5; 4.6)	61	3.9 (2.7; 5.1)	−4 (−25; 17)	5 (−15; 25)	0.50	0.43
Ukraine	56	3.4 (2.1; 4.6)	53	3.2 (2.6; 4.4)	60	3.5 (2.2; 4.8)	3 (−148; 22)	6 (−116; 25)	0.51	0.44
Combined	162	3.4 (2.4; 4.5)	184	3.6 (2.5; 4.9)	178	3.2 (2.4; 4.5)	8 (−9; 25)	0 (−24; 25)	0.90	0.73
Total count ($\times 10^6$)[d]										
Greenland	59	152 (72; 316)	58	192 (79; 334)	58	205 (78; 345)	−5 (−40; 31)	4 (−33; 41)	0.40	0.76
Poland	58	171 (68; 417)	61	210 (109; 513)	60	215 (115; 465)	57 (11; 103)*	33 (−11; 77)	0.79	0.26
Ukraine	56	193 (95; 370)	53	204 (115; 389)	60	192 (102; 327)	14 (−21; 51)	−5 (−41; 30)	0.61	0.77
Combined	162	197 (99; 371)	183	197 (93; 452)	178	186 (75; 340)	23 (−11; 57)	18 (−32; 67)	0.33	0.67
Percent motile sperm[e]										
Greenland	63	60 (40; 69)	65	61 (43; 72)	65	58 (43; 68)	−5 (−24; 13)	−5 (−24; 14)	0.83	0.96
Poland	35	60 (47; 75)	45	68 (57; 75)	37	68 (56; 76)	11 (−6; 28)	−4 (−20; 13)	0.27	0.70
Ukraine	56	58 (41; 74)	60	62 (42; 74)	62	56 (45; 74)	6 (−13; 26)	7 (−12; 26)	0.83	0.24
Combined	164	61 (43; 75)	133	63 (51; 74)	191	60 (43; 70)	1 (−16; 18)	−1 (−26; 25)	0.41	0.27
Percent normal cells										
Greenland	65	7 (4; 10)	65	7 (4; 10)	65	6 (4; 10)	3 (−20; 25)	−2 (−25; 21)	0.89	0.82
Poland	62	6 (4; 10)	63	6 (4; 10)	64	6 (4; 8)	−9 (−35; 17)	−19 (−45; 6)	0.10	0.10
Ukraine	67	8 (4; 11)	68	8 (4; 10)	67	6 (4; 10)	−3 (−26; 20)	−17 (−40; 6)	0.11	0.08
Combined	194	8 (4; 10)	196	6 (4; 10)	196	6 (4; 10)	−22 (−44; −1)*	−35 (−66; −4)*	0.14	0.06

Adjusted difference from the first tertile represents percentage change.
[a]Adjusted for age, abstinence time, spillage, smoking, urogenital infections and BMI. Combined analyses were adjusted for country as well.
[b]Spearman rank correlation.
[c]Multiple linear regression adjusted for the above-mentioned potential confounders (ln-transformed exposure and outcome).
[d]Restricted to samples from men reporting no spillage.
[e]Restricted to samples analysed within 1 h after semen collection.
*Significant differences ($P < 0.05$) compared with the reference group.

non-significant decrease in the proportion of normal sperm was also observed at the second tertile. Also PFNA was associated with a non-significant lower proportion of normal sperm at higher exposure, but none of the associations were statistically significant in analyses based on continuous exposure.

Discussion

The present study indicates that PFC exposure may be associated with alterations in human semen quality. The most pronounced effect observed was an increase in sperm cell morphology defects at increasing PFOS exposure in the two European populations but not in the Inuit population from Greenland. Analyses on specific morphological outcomes did not identify effects of PFOS on specific parts of sperm morphology, suggesting small alterations in several aspects of sperm morphology. A higher proportion of motile sperm at higher PFOA exposure emerged in this study, but this association might be driven solely by the observed association in Greenland and was not supported by similar effects in the other countries.

The present study is the largest one to date investigating the associations between PFC exposure and human semen quality. Two previous studies have been published evaluating the semen quality in relation to PFC exposure among 105 young Danish men (Joensen *et al.*, 2009) and 256 American infertility clients (Raymer *et al.*, 2011). In these studies no statistically significant association between PFC exposures and sperm concentration, volume or motility could be detected, but in the Danish study, a combined estimate of high PFC exposure based on the sum of quartile scores of PFOS and

Table IV Associations of PFOS to specific aspects of sperm morphology combined from the three countries.

Percentage of cells with	First tertile (<11.9 ng/ml) Median (P25; P75); *n* = 194	Second tertile (11.9–27.2 ng/ml) Median (P25; P75); *n* = 196	Third tertile (>27.2 ng/ml ng/ml) Median (P25; P75); *n* = 196	Adjusted difference from first tertile (95% CI)[a]		Trend test	
				Second tertile	Third tertile	*P* crude[b]	*P* adjusted[c]
Head defect	92 (88; 96)	93 (90; 96)	93 (90; 95)	1.0 (−0.5; 2.5)	1.6 (−0.6; 3.8)	0.22	0.20
Midpiece defect	17 (14; 21)	17 (14; 20)	18 (16; 23)	4.7 (−5.7; 15.1)	8.8 (−6.2; 24.0)	<0.01*	0.37
Tail defect	6 (4; 10)	7 (5; 12)	6 (4; 9)	28.6 (7.3; 49.8)*	22.3 (−8.4; 53.1)	0.98	0.24
Cytoplasm drop	3 (2; 4)	2 (1; 3)	2 (1; 3)	13.6 (−7.9; 39.8)	7.5 (−24.8; 39.8)	<0.01*	0.74
Immature spermatozoa	1 (1; 2)	1 (0; 2)	2 (1; 3)	9.6 (−21.1; 40.3)	27.9 (−17.6; 73.3)	0.80	0.73

Adjusted difference from the first tertile represents percentage change.
[a]Adjusted for age, abstinence time, spillage, smoking, urogenital infections, BMI and country.
[b]Spearman rank correlation.
[c]Multiple linear regression adjusted for the above-mentioned potential confounders (ln-transformed exposure and outcome).
*Significant associations ($P < 0.05$).

PFOA indicated that high PFC exposure was associated with a lower proportion of normal sperm (Joensen *et al.*, 2009). Sperm cell morphology was unfortunately not evaluated in the American study (Raymer *et al.*, 2011). Thus, our finding of a significant association between PFOS exposure and the proportion of normal sperm is in line with results from the previous Danish study, and further indicates that the association between PFC exposure and normal sperm is more likely to be caused by exposure to PFOS than to PFOA.

The average concentrations of PFCs in the present study are similar to the previous Danish study with a median of 24.5 ng/ml PFOS and 4.9 ng/ml PFOA (Joensen *et al.*, 2009), and slightly lower compared with the American study with a median of 32.3 ng/ml PFOS and 9.2 ng/ml PFOA (Raymer *et al.*, 2011).

In our study we also observed a decreased proportion of normal sperm at higher levels of PFHxS. However, due to the high correlation between PFOS and PFHxS ($r = 0.91$), the association between PFHxS and sperm morphology may be driven by effects of PFOS, which is measured at an ~20-fold higher concentration in the studied men. The lack of independent effect of PFHxS is further supported by a supplementary analysis with mutual adjustment for PFOS, PFOA and PFNA indicating no association between PFHxS and normal sperm when adjusted for other PFC exposures, but a similar overall association between PFOS exposure and the percentage of normal sperm (data not shown).

The potential mechanism by which PFOS may affect sperm morphology is not fully understood. Animal studies indicate PFOS exposure may affect the transcription of inhibins and steroidogenic enzymes (StAR, CYP11A1, CYP17A1, 3β- and 17β-honest significant difference; Zhao *et al.*, 2010; Wan *et al.*, 2011). However, a recently published study on the same population did not indicate alteration in reproductive hormones in relation to PFC exposure (Specht *et al.*, 2012). Also sperm DNA integrity measured by sperm chromatin structure assay or by *in situ* terminal deoxynucleotidyl transferase dUTP nick-end labelling assay together with the expression of apoptotic markers in semen (Fas-receptor and Bcl-xL) were not consistently, across countries, associated with PFCs (Specht *et al.*, 2012), suggesting that the effects of PFOS on sperm morphology are likely to be specific effects occurring in the cell membranes. Due to the amphiphilic properties of PFCs they tend to bind to cell membranes, and especially PFOS has been shown to cause alterations in membrane properties (Hu *et al.*, 2003). It can be speculated that specific effects on sperm morphology may be caused by PFOS-induced alterations in sperm cell membrane properties and in such a manner affect the shape of the sperm.

The observed positive association between PFOA exposure and the proportion of motile sperm has not been observed in previous human studies (Joensen *et al.*, 2009; Raymer *et al.*, 2011), and it was only evident in one out of our three populations in the present study. The positive association between PFOA and sperm motility may thus represent a chance finding.

Due to the numerous statistical tests conducted in the present study, the observed association between PFOS exposure and the proportion of normal sperm may also have been found by chance, but consistency with the previous study (Joensen *et al.*, 2009) and the fairly consistent magnitude of associations between countries makes it less likely to be a spurious finding.

One may consider analysing the results in relation to a combined score of PFC exposure, if the studied PFCs can be considered as having similar effects on semen quality. However, our results do not suggest similar associations between exposure to different PFCs and the studied outcomes. Furthermore, combining PFCs for risk assessment was not recommended in a study specifically aimed at evaluating whether combined PFC exposure could be used in risk assessment (Scialli *et al.*, 2007).

Of the so far measured compounds, the highest correlation was found for the polychlorinated biphenyl 153 (PCB-153), which was only moderately correlated with PFOS, PFHxS and PFNA ($r = 0.4–0.5$) but not correlated with PFOA ($r = 0.04$). Furthermore, in the same study population a negative association between sperm motility and PCB-153 exposure has been observed, but the proportion of normal sperm cells were not associated with PCB-153 or p,p′-DDE exposure (Toft *et al.*, 2006). Therefore, the observed associations are not likely to be caused by the other compounds measured in the serum.

The participation rates in the present study varied considerably between populations and, especially in Ukraine and Poland, potential

Table V Semen quality in relation to PFOA exposure in tertiles and continuous trend tests.

Parameter	First tertile		Second tertile		Third tertile		Adjusted difference from first tertile (95% CI)[a]		Trend test	
	n	Median (P25; P75)	*n*	Median (P25; P75)	*n*	Median (P25; P75)	Second tertile	Third tertile	*P* crude[b]	*P* adjusted[c]
Sperm concentration (10^6/ml)										
Greenland	65	49 (28; 82)	65	53 (29; 99)	66	62 (36; 98)	11 (−18; 41)	22 (−9; 52)	0.28	0.18
Poland	63	58 (25; 103)	63	58 (33; 123)	63	83 (39; 122)	3 (−36; 43)	22 (−19; 64)	0.20	0.94
Ukraine	67	64 (35; 97)	68	53 (34; 95)	68	56 (33; 99)	−7 (−36; 23)	−3 (−33; 27)	0.77	0.69
Combined	196	59 (33; 96)	195	57 (28; 99)	197	63 (36; 114)	4 (−27; 36)	15 (−17; 48)	0.15	0.45
Volume (ml)[d]										
Greenland	60	3.2 (2.2; 4.9)	61	3.4 (2.4; 4.4)	54	3.4 (2.6; 4.7)	2 (−17; 21)	6 (−14; 26)	0.26	0.09
Poland	57	3.5 (2.7; 4.7)	61	3.2 (2.4; 4.5)	62	3.8 (2.6; 5.1)	−1 (−21; 19)	7 (−14; 28)	0.53	0.73
Ukraine	55	3.4 (2.1; 4.2)	53	3.5 (2.6; 4.5)	61	3.4 (2.4; 4.9)	4 (−15; 22)	8 (−11; 26)	0.57	0.38
Combined	163	3.2 (2.2; 4.5)	181	3.4 (2.4; 4.8)	180	3.5 (2.6; 4.7)	3 (−16; 22)	10 (−10; 29)	0.20	0.07
Total count ($\times 10^6$)[d]										
Greenland	60	189 (85; 299)	61	174 (72; 346)	54	218 (80; 350)	1 (−34; 37)	22 (−16; 59)	0.26	0.06
Poland	57	168 (82; 434)	61	201 (89; 417)	61	238 (122; 537)	20 (−25; 65)	43 (−3; 91)	0.15	0.44
Ukraine	55	174 (97; 340)	53	233 (84; 370)	61	196 (110; 410)	−5 (−42; 31)	0 (−37; 36)	0.91	0.95
Combined	163	196 (94; 370)	181	182 (84; 340)	179	215 (107; 419)	4 (−34; 42)	22 (−17; 62)	0.27	0.34
Percent motile sperm[e]										
Greenland	64	55 (36; 68)	64	62 (42; 70)	65	61 (47; 71)	10 (−8; 29)	36 (6; 45)*	0.03*	0.01*
Poland	40	58 (45; 69)	39	67 (61; 78)	38	72 (59; 79)	7 (−9; 23)	14 (−3; 30)	0.01	0.32
Ukraine	58	63 (41; 75)	60	54 (40; 74)	60	56 (48; 71)	−6 (−25; 12)	10 (−10; 29)	0.46	0.06
Combined	172	57 (41; 74)	159	58 (43; 70)	157	65 (53; 73)	3 (−15; 21)	19 (1; 39)*	0.01*	0.01*
Percent normal cells										
Greenland	65	6 (4; 9)	65	8 (4; 10)	65	7 (4; 10)	15 (−7; 37)	16 (−7; 39)	0.33	0.24
Poland	63	6 (3; 9)	63	6 (4; 8)	63	8 (4; 10)	0 (−25; 25)	19 (−8; 46)	0.18	0.45
Ukraine	67	8 (4; 10)	68	8 (4; 10)	67	7 (4; 10)	−6 (−29; 17)	0 (−24; 24)	0.66	0.94
Combined	196	8 (4; 10)	194	6 (4; 10)	196	7 (4; 10)	−5 (−28; 18)	10 (−13; 34)	0.89	0.50

Adjusted difference from the first tertile represents percentage change.
[a]Adjusted for age, abstinence time, spillage, smoking, urogenital infections and BMI. Combined analyses were adjusted for country as well.
[b]Spearman rank correlation.
[c]Multiple linear regression adjusted for the above-mentioned potential confounders (ln-transformed exposure and outcome).
[d]Restricted to samples from men reporting no spillage.
[e]Restricted to samples analysed within 1 h after semen collection.
*Significant associations ($P < 0.05$).

selection bias could be an issue, since men participating in the study who provided a semen sample had slightly but not statistically significantly longer times to pregnancy compared with men not delivering a semen sample (Toft *et al.*, 2005a). Furthermore, since the men in the studies have a pregnant partner, we did not include sterile men and highly subfertile men will be underrepresented. None of the men included in the study knew about their exposure to PFCs when deciding to participate and therefore differential selection is unlikely. However, it should be acknowledged that our conclusions are limited to fertile men from the general population.

The exposure in the present study is measured with a highly sensitive LC/MS/MS technique and PFOS, PFOA, PFNA, PFHxS could be detected in almost all samples. Also, due to the long half-life of PFOS and PFOA in humans (Olsen *et al.*, 2007), one sample is a good estimate of the exposure at least during the period of spermatogenesis (~64 days; Heller and Clermont, 1963). Therefore, we do not expect major misclassification of exposure.

Male semen quality measures are known to vary considerably from day to day (Keel, 2006). Furthermore, the estimation of semen quality may vary between technicians before standardized training (Bjorndahl *et al.*, 2002). Therefore, a special quality assurance program was included in the present study with training of technicians before and during semen analysis to evaluate the variability of the obtained results between observers. We obtained a low degree of interobserver variation (CV <11%) for sperm cell motility and concentration, and no significant differences between technicians, indicating that potential differences between countries (representing different exposures) are not likely to be due to interobserver variability (Toft *et al.*, 2005b). Thus, although some misclassification of outcomes is expected, it is not likely to be associated with the exposure, and

Table VI Semen quality in relation to PFHxS and PFNA exposure combined from the three countries in tertiles and continuous trend tests.

Parameter	First tertile		Second tertile		Third tertile		Adjusted difference from first tertile (95% CI)[a]		Trend test	
	n	Median (P25; P75)	*n*	Median (P25; P75)	*n*	Median (P25; P75)	Second tertile	Third tertile	*P* crude[b]	*P* adjusted[c]
Sperm concentration (mill/ml)										
PFHXS	196	60 (33; 97)	195	59 (30; 110)	197	58 (30; 100)	−12 (−52; 28)	−11 (−57; 35)	0.22	0.54
PFNA	196	59 (31; 98)	196	54 (31; 102)	196	63 (35; 104)	−1 (−19; 18)	7 (−13; 28)	0.42	0.53
Volume (ml)[d]										
PFHxS	161	3 (2; 5)	183	4 (2; 5)	180	3 (2; 5)	6 (−17; 29)	8 (−19; 34)	0.83	0.24
PFNA	167	4 (2; 5)	178	3 (2; 5)	179	3 (2; 4)	0 (−11; 12)	−5 (−17; 7)	0.35	0.34
Total count (mill)[d]										
PFHxS	161	197 (101; 390)	182	191 (93; 420)	180	189 (77; 345)	−5 (−51; 41)	−6 (−59; 48)	0.07	0.82
PFNA	167	204 (88; 407)	178	187 (86; 399)	178	195 (104; 340)	2 (−21; 24)	5 (−19; 29)	0.99	0.93
Percent motile sperm[e]										
PFHxS	169	58 (41; 74)	138	64 (51; 74)	181	60 (43; 71)	11 (−12; 35)	10 (−18; 37)	0.56	0.53
PFNA	159	63 (44; 75)	161	59 (46; 72)	168	60 (43; 72)	0 (−12; 11)	−1 (−13; 11)	0.13	0.34
Percent normal cells										
PFHxS	195	8 (4; 10)	195	6 (4; 10)	196	6 (4; 10)	−27 (−58; 3)	−35 (−70; −1)*	0.13	0.20
PFNA	195	7 (4; 10)	195	6 (4; 10)	196	6 (4; 10)	−12 (−26; 2)	−8 (−23; 7)	0.30	0.36

Adjusted difference from the first tertile represents percentage change.
[a]Adjusted for age, abstinence time, spillage, smoking, urogenital infections, BMI and country.
[b]Spearman rank correlation.
[c]Multiple linear regression adjusted for the above-mentioned potential confounders (ln-transformed exposure and outcome).
[d]Restricted to samples from men reporting no spillage.
[e]Restricted to samples analysed within one hour after semen collection.
*Significant associations ($P < 0.05$).

therefore, if any effect, it would most likely bias the results towards no effect.

We *a priori* included a number of potential confounders in the present study. Distributions of several of the included confounders vary considerably between countries. Since exposure also varies considerably between countries, the overall studied effects across countries may reflect differences between countries to some extent and caution should be taken in interpreting the results across populations. By including country as a potential confounder in the analyses across countries, we expect to capture most of these country-related differences, but residual confounding by other factors differing between countries cannot be excluded. However, adjusting for country might overadjust for potential effects of PFOS since the exposure to PFOS is markedly different between countries. Therefore, a subanalysis without adjustment for country was performed. This analysis showed similar but not stronger associations between PFOS and the percentage of normal sperm indicating that we did not overadjust by including country as a confounder in our analysis (data not shown).

In conclusion, PFCs may be associated with alterations in the human semen quality, although sperm concentration [the most important determinant of male fertility (Bonde *et al.*, 1998)] was not affected. The results of the present study were in line with the findings of effects of PFCs on sperm morphology reported in a previous smaller study (Joensen *et al.*, 2009), and we further identified PFOS as a potential causative agent.

Supplementary data

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

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Authors' roles

G.T. contributed to the study design and data collection, and analyzed data and drafted the manuscript. B.A.G.J. and C.L. collected data and contributed to the study design. V.L. and R.V. contributed to the study design and data analyses. M.S., D.H. and L.R., A.G. and J.P.B. contributed to the study design. H.S.P., J.K.L. and V.Z. contributed to data collection. All authors revised the manuscript critically and approved the final version for publication.

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Conflict of interest

None declared.

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