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Serum levels of perfluorinated compounds and sperm Y:X chromosome ratio in two European populations and in Inuit from Greenland

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Abstract

This study investigated whether perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS), which exhibit reproductive toxicity in experimental animals, affect sperm sex chromosome ratio.

The Y:X ratio was determined by fluorescence *in situ* hybridization. Serum concentrations of PFOA and PFOS were measured in 607 men from Greenland, Poland and Ukraine using liquid chromatography-tandem mass spectrometry. Data was analyzed by linear and nonlinear regression.

We observed no associations between PFOA and Y:X ratio ($p=0.845$ in a linear model, $p=0.296$ in a nonlinear model). A positive nonlinear association between PFOS and Y:X ratio was observed ($p=0.016$), with no association in a linear model ($p=0.118$). Analyzing the populations separately, a negative trend between categorized PFOS exposure and Y:X ratio was observed for the Inuit ($B=-0.002$, $p=0.044$).

In conclusion, there was a negative trend between Y:X ratio and PFOS in the Inuit, while there was no association between PFOA and the Y:X ratio in adult men.

1. Introduction

Worldwide the rate of male to female births is 1.07, which corresponds to a sex birth ratio of 0.517 (www.cia.gov). Factors known to influence the genetic sex of the newborn are: maternal age [1], birth order of the child [2] and stress due to natural disasters [3] or war [4]. Other factors, such as ethnicity [5, 6], length of follicular phase [1, 7, 8] and, diabetes [7] are also suspected to change the sex ratio.

A declining male:female sex ratio of newborns has been reported [9, 10]. It has been suggested that hormonal imbalances in the mother or father may be related to offspring gender [5, 11], or that substances with teratogenic or mutagenic properties affect the normal sex determination of the embryo [12]. Environmental pollutants are regarded as yet another contributing factor to the decline in sex birth ratio, as evidenced by the accident in 1976 in Seveso, Italy, where young men were exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) subsequently fathered more girls than boys [13]. Other authors have pointed out that initial reports on change in offspring sex ratio seldom are corroborated [14, 15] and that the easy access to data on child sex in large datasets may be related to a high rate of false positive reports as a result of publication bias [16].

Perfluoroalkyl substances (PFAS), such as perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS) are anthropogenic ubiquitous breakdown products of substances used for many commercial purposes, and they are highly persistent in nature [17-20]. Also animals like polar bears, seals and bald eagles living in the Arctic and Alaska have been shown to have these chemicals in their circulation [21] due to atmospheric and oceanic current transports [20, 22].

Biomonitoring data of PFOA and PFOS from general population in the US show that PFOS concentration in blood has dropped from between 30-40 ng/ml in the year 2000 to between 12-14 ng/ml in 2008 [23] and 8-10 ng/ml in 2010 [24, 25]. Also, PFOA concentration in blood have seen a small decrease from between 4-5 ng/ml to 2-4 ng/ml during the same period. These findings agree with biomonitoring collected between 2005-2009 from several European countries and from China [26-30]. Occupational exposure to these compounds is however markedly different. A study on male workers involved in ammonium perfluorooctanoate production showed that mean serum PFOA, measured in 1993, 1995 and 1997, was 5000 (SD 12200) ng/ml, 6800 (SD 16000) ng/ml and 6400 (SD 14300) ng/ml [31]. Another study looking at male volunteers from the 3M company workforce at three different locations found mean serum PFOA and PFOS concentrations at 2.21 (SD 6.40) µg/ml and 1.05 (SD 0.97) µg/ml respectively [32].

The main exposure routes to PFAS by humans is ingestion of food of animal origin, food that has been in contact with PFAS-treated wrappings, drinking water and household dust [33-37]. It should however be noted that the European Food Safety Authority in 2012 concluded that exposure estimates in all age classes and for both mean and the 95th percentile consumers were well below the TDIs (tolerable daily intake) for both PFOS (150 ng/kg b.w. per day) and PFOA (1500 ng/kg b.w. per day) [36].

Both PFOA and PFOS are classified as endocrine disrupting compounds (EDCs) [38].

Negative associations of these compounds were demonstrated by finding that among 100 Danish military conscripts, those with high serum concentrations of PFOA and PFOS had a reduced number of morphologically normal spermatozoa compared to those with low serum concentrations [39]. Other studies show no effect of PFAS exposure on sperm

parameters [40-42]. Despite numerous toxicological and epidemiological studies that have examined reproductive and developmental associations [42-47], none has reported on sex ratios. Therefore our objective was to investigate whether exposure to certain PFAS, reflected by serum levels, can influence the proportion of Y-chromosome bearing sperm cells in samples collected from two European populations as well as from the Inuit population on Greenland.

2. Materials and methods

2.1 Study population

Male spouses of pregnant women in Greenland, Poland (Warsaw) and Ukraine (Kharkiv) were recruited for a study on the impact of exposure to persistent organic pollutants on reproductive function [48]. In all three countries, pregnant women were approached as the entry point for the study, and their male spouses were then enrolled in the study from May 2002 throughout February 2004. To be included in the study, both the male and his female partner had to be at least 18 years of age.

In Greenland, 256 male partners to pregnant women were asked to participate in the semen study. In Warsaw and Kharkiv, 690 and 640 male partners to pregnant women, respectively, were asked to enroll. [48]. Blood and semen samples were collected from 201 men from Greenland (79% participation rate), 198 from Warsaw (29% participation rate), and 208 from Kharkiv (33% participation rate). The reasons for denying taking part in the study were not recorded. The subjects were asked to abstain from sexual activities for at least 48 hours before collecting the sample and to note the actual abstinence time.

Information on lifestyle was collected through interviews.

The study was approved by local ethical committees and all subjects signed an informed consent.

2.2 Analysis of PFAS

The analysis of PFOA and PFOS in the sera was performed as previously described [49]. Briefly, labeled internal standards were added and the proteins were precipitated by organic solvent. The supernatant was injected on a liquid chromatography-tandem mass

spectrometry (LC/MS/MS). In all sample batches, the quality of the measurements was controlled by analyzing chemical blanks and in-house quality control samples. The analyses were part of the Erlangen Round Robin inter-laboratory control program. The limits of detection and reproducibility have been described elsewhere [49].

2.3 *Fluorescent in situ hybridization*

Semen samples were analyzed by a two color fluorescence *in situ* hybridization (FISH) assay, as has previously been described in detail in Tiido *et al* 2005 [50].

Briefly, semen samples were smeared on glass slides where they were labeled using protein-nucleic acid probes specific to either the centromeric region of the X chromosome or the q-arm of the Y-chromosome. The slides were examined under a fluorescent microscope and Y or X chromosome in a sperm nucleus was recognized by a red or a green fluorescent spot, respectively. Sperm nuclei were only scored when morphologically preserved, not clumping or overlapping, showing a well-defined outline and tail and with a sperm head decondensed to no more than twice the size of normal non-decondensed spermatozoa. Sperm showing several signals (disomic or diploidic) were not counted. Sperm cells showing normal decondensation, but without X or Y signals, were scored and considered valid for calculation of the hybridization efficiency.

In all but 6 cases, ≥ 500 cells/sample were scored. According to a quality control program, the inter- and intra-observer coefficient of variation regarding the proportion of Y chromosome bearing sperm cells was 2.3% and 3.3%, respectively.

2.4 *Statistical analysis*

The Y:X chromosome ratio was calculated as the mean \pm SD Y:X ratio of all samples that fulfilled the inclusion criteria [51].

In order to explore the shape of possible associations between exposures and proportions of the sex chromosomes, the overall relationships between PFOA, PFOS and the Y:X chromosome ratio were first investigated by thin plate regression spline analysis [52].

If the spline analysis was not obvious incompatible with a linear relationship, linear regression analysis was undertaken. Exposure levels were also categorized into quartiles (25th, 50th and 75th percentiles) and analyzed by multivariate analysis of variance to further explore non-linear relationship and exposure threshold levels.

All three populations were analyzed together, as well as separately, but an interaction analysis between Y:X chromosome ratio, PFOA or PFOS exposure levels and population was included in order to ascertain possible center-dependent heterogeneity in the exposure-sex chromosome ratio association.

All analyzes were done twice; first with the original dataset and then repeated with a dataset where the most extreme and influential outliers had been removed. The modified datasets excluded any data points that fulfilled any of the following criteria: PFOA level in serum ≥ 20 ng/ml (n=1); Abstinence time ≥ 21 days (n=9); 2,2',4,4',5,5'-hexachlorobiphenyl (CB-153) level in serum ≥ 15 ng/ml (n=1).

Confounding factors that were corrected for in all calculations were age, abstinence time, alcohol intake and CB-153 as an earlier study, using the same population, showed that CB-153, but not dichlorodiphenyldichloroethene (*p,p'*-DDE), correlated negatively with the proportion of Y-chromosome bearing sperm cells in Poland [51].

Results are presented as either p-values or p-values and adjusted r^2 . For trend analysis results are expressed as p-values, B-values and 95 % confidence intervals for B. A p-value of 0.05 was adopted as the level of statistical significance for all statistical analyses. For the spline analysis, the mgcv-package developed for R was used (R Development Core Team 2010, R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL: <http://www.R-project.org/>) and for all other analyses SPSS (SPSS v19 statistical software, SPSS, Inc., Chicago, IL, USA) was used.

3. Results

3.1 Fluorescence *in situ* hybridization

Of the 607 samples, 98 were excluded as not enough semen was available for fluorescence *in situ* hybridization analysis. Ninety five samples were excluded because of too low number of sperm cells (<500) or failure during analysis and an additional 55 samples were excluded from the analysis as exposure data was unavailable. The final number of participants was thus 359 (22.6 % participation rate). Apart from sperm counts, which were higher in included than excluded samples in all populations, the Ukrainian men that were included also had longer abstinence time ($p=0.024$), whereas the Polish men had higher sperm motility ($p=0.011$) and higher CB-153 levels ($p=0.011$, Table 1).

The Y:X chromosome ratios for the 359 participants were on average significantly higher in Greenland (0.513) compared to both Poland (0.503; $p<0.001$) and Ukraine (0.508; $p=0.013$), and in Ukraine compared to Poland ($p=0.042$).

3.2 Spline analysis

Spline analysis of the pooled dataset showed a positive association between Y:X chromosome ratio and serum concentration of PFOS ($p=0.005$, adjusted $r^2=0.054$, Table 2, Fig 1), whereas no association was present with PFOA. However, when each population was analyzed separately, an association between high levels of PFOA and higher Y:X chromosome ratio was found in the Ukrainian population ($p=0.015$, adjusted $r^2=0.045$). When the most extreme outliers were excluded (Fig 2A and B), the spline analysis on the pooled dataset still showed a positive association between Y:X chromosome ratio and exposure to PFOS ($p=0.016$, adjusted $r^2=0.057$). However, country-wise no association between PFOA or PFOS exposure and Y:X chromosome ratio was present.

3.3 Linear regression analysis

Linear regression analysis of the pooled dataset revealed associations for both PFOS ($p=0.026$, adjusted $r^2=0.016$, Table 2) and PFOA ($p=0.050$, adjusted $r^2=0.013$) with Y:X chromosome ratio. Country-wise, as in the spline analysis, there was a positive association between PFOA exposure and the Y:X chromosome ratio in Ukraine ($p=0.017$, adjusted $r^2=0.019$).

After exclusion of extreme outliers the linear regression analysis showed no statistically significant associations, neither country-wise, nor for the populations taken together.

3.4 Multivariate analysis of variance

In the multivariate analysis of variance model, when grouping the three populations together as one, there was a significant difference in Y:X chromosome ratio between the 2nd and the 4th quartile ($p=0.006$, Table 3) of categorized PFOS exposure, but no differences were found for PFOA. A trend analysis showed a positive linear association between increasing PFOS exposure and increasing Y:X chromosome ratio in the three populations combined ($B=0.002$; $p=0.017$; 95 % CI for $B=0.000 - 0.004$; Table 4). Also, significant differences were found between the 2nd and the 4th quartile ($p=0.042$) as well as between the 3rd and the 4th quartile ($p=0.036$) of categorized PFOS exposure in the Inuit population. A trend analysis on this population showed a negative association between PFOS exposure and Y:X chromosome ratio ($B=-0.002$; $p=0.028$; 95 % CI for $B=-0.004 - 0.000$). No statistically significant differences were found when these analyses were performed on the Polish or Ukrainian populations. The interaction analysis showed a

significant inter-center difference ($p < 0.001$) in how the Y:X chromosome ratio was affected by the PFOS exposure, i.e. the association between exposure and Y:X chromosome ratio differed depending on which of the three populations that was studied. After exclusion of extreme outliers, a trend analysis still showed a positive association between increasing PFOS exposure and increasing Y:X chromosome ratio in the three populations combined ($B = 0.002$; $p = 0.039$; 95 % CI for $B = 0.000 - 0.004$). No associations were found with PFOA. The multivariate analysis of variance model showed a difference in Y:X chromosome ratio between the 2nd and the 4th quartile of PFOS exposure ($p = 0.043$). A trend analysis still showed a negative association between PFOS exposure and Y:X chromosome ratio ($B = -0.002$; $p = 0.044$; 95 % CI for $B = -0.004 - 0.000$) in the Inuit population. No differences were found within the Polish or Ukrainian populations. The interaction analysis still showed a statistically significant ($p < 0.001$) inter-center heterogeneity.

4. Discussion

In this study we investigated the relationship between sperm Y:X chromosome ratio and PFOA or PFOS exposure, reflected by their serum levels, in two mainland European populations as well as in the Inuit population on Greenland. Our main findings were a positive linear trend between Y:X chromosome ratio and exposure to PFOS, as well as the lack of any associations between PFOA and Y:X chromosome ratio. This may reflect differences in exposure levels across the three populations or an ecological bias that arises due to regional differences in PFOA or PFOS exposure effect on the populations as the interaction analysis indicated an inter-center heterogeneity.

Another finding was a negative linear trend between categorized PFOS exposure and Y:X ratio in the Inuit population.

Following exclusion of extreme values, it was only in Greenland that any statistically significant associations were found when analyzing the populations separately. However, the findings in the Inuit population, both before and after exclusion of outliers, depend upon a drop in Y:X ratio between the 3rd and 4th quartile of categorized PFOS exposure. The lack of significant associations in Poland and Ukraine might be due to insufficient statistical power or less exposure contrast within countries compared to the exposure contrast between countries.

Several studies have highlighted the endocrine disrupting effects of PFAS. In *Cynomolgus* monkeys, exposure to 0.75 mg PFOS/kg/day for 183 days resulted in decreased estradiol and thyroid hormone levels in serum [53]. It should be noted though that at the end of the 183 days, serum concentrations of PFOS approximated 170 µg/ml, which is 5800 times more than in current work and hence incomparable with the levels discussed in this study.

Also, in rare minnow fish, PFOA exposure inhibited expression of genes responsible for thyroid hormone biosynthesis and also induced estrogen responsive genes [54]. Rats exposed to 25 mg PFOA/kg/day for 14 days developed Leydig cell hyperplasia and eventually also Leydig cell adenomas [55, 56]. Male rats exposed to 5 or 10 mg of the PFAS perfluorododecanoic acid/kg/day for 14 days exhibited a decrease in testosterone levels and an increase in estradiol levels [57]. However, these rats also suffered a significant body weight loss and subsequently also smaller testis weights, which may explain the hampered testosterone secretion. Lowered testosterone levels and Leydig cell hyperplasia are common features among infertile men [58, 59] and higher levels of PFOA and PFOS has previously been shown in infertile men [60]. On the other hand, there are also studies showing no adverse PFOA or PFOS-effects on semen parameters in neither men [41] nor rats [42]. In another study on rats given 3 mg potassium PFOS/kg body weight for 7 consecutive days, no functional alteration in the hypothalamic-pituitary-thyroid axis was noted [61]. Chang et al 2007 also showed that short term exposure to PFOS did not reduce thyroid hormone levels in female rats [62].

The Y:X chromosome ratios in this study differed between the Inuit men (0.513) and the men in both Poland (0.503) and Ukraine (0.508). A previous study, using the same populations, found Y:X chromosome ratios of 0.512 for the Inuit, 0.503 for Poland and 0.507 for Ukraine [51]. This small difference in Y:X ratio between the studies can be explained by the inclusion of slightly different groups of men, as different individuals had to be left out due to lack of exposure data. Although the Y:X ratios differ between the populations, for unknown reasons, their sex birth ratios are similar, 0.512 for Greenland, and 0.515 for both Poland and Ukraine. The Polish and Ukrainian populations have a low

sex birth ratio compared to their Y:X chromosome ratio when matched to the Inuit. This difference in Y:X ratio but not in sex birth ratio may be due to a female factor or a differing inter-population exposure pattern to other environmental chemicals than PFOA and PFOS which affect the balance between Y:X chromosome ratio and sex birth ratio in humans. In rats, full scale 2-generation studies with PFOS [63] and PFOA [64] did not report any deviations in male/female offspring numbers.

The rather low participation in the semen study might, to some degree, affect the results. However, as all included men were proven fertile, no bias could have arisen from men participating because of fear of infertility. Also, the great difference in participation rate between the Inuit and the mainland populations could mean that the Inuit were better represented than the Europeans were.

The selection of the included samples might, to some degree, affect the results. The three cohorts included are not representative of the general population, as they were recruited for their proven fertility. Both these factors might affect the individual susceptibility to POPs. Furthermore, the genetic makeup of the Greenlandic Inuit is somewhat different from that of Caucasians. For example, it has been shown that the Greenland Inuit are genetically predisposed to a lower risk of cardiovascular disease [65] and to a lower risk of hypospadias than Europeans [66]. Another potential bias for this study was the lack of hybridization signal in up to 5% of the spermatozoa because of insufficient hybridization. However, a hybridization efficiency of 95% or more is in good accordance with the hybridization efficiency previously reported [67, 68].

In conclusion, when analyzing the three countries together, we found a positive linear trend between PFOS exposure and Y:X ratio, but no association between PFOA and Y:X ratio.

Due to inter-center heterogeneity, the populations were analyzed separately and the only association found on single population level was in the Inuit, where there was a negative trend, between categorized PFOS exposure and Y:X ratio. The find in the Inuit population, or the lack of finds in the Polish and Ukrainian populations, might be due to insufficient power. Due to the inter-center heterogeneity with regard to how the exposure levels of PFOS affect the Y:X chromosome ratio, we cannot draw firm conclusions based on the compiled material. Nevertheless, it seems that exposure to PFOA or PFOS does not have an adverse effect on the Y:X chromosome ratio.

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Legends to figures

Figure 1. Explorative spline analysis

Explorative spline analysis showing the association between Y:X ratio and PFOS exposure in the Inuit (blue), Polish (green) and Ukrainian (black) populations taken together. The shaded area represents 95% CI.

Figure 2. Scatter plots of PFOS and PFOA exposure

Scatter plots of PFOS (A & B) and PFOA (C) exposure with Y:X chromosome ratio in the Inuit (blue, solid regression line), Polish (green, dotted regression line) and Ukrainian (black, solid regression line) population, outliers are marked with red. 95% CI for the population specific regression lines are shown as shaded areas.

Table 1. Background characteristics for samples included as well as excluded from the FISH analysis. Values are shown as mean (95% CI)

Background characteristics	Greenland			Poland			Ukraine		
	Included (n=161)	Excluded (n=40)	p	Included (n=122)	Excluded (n=76)	p	Included (n=131)	Excluded (n=77)	p
Age (years)	30.9 (29.9-32.0)	31.4 (29.0-33.8)	0.694	30.3 (29.6-31.0)	30.3 (29.3-31.2)	0.908	26.0 (25.1-26.9)	27.4 (26.1-28.7)	0.087
Abstinence time (days)	5.70 (2.43-8.96)	3.67 (2.66-4.68)	0.543	6.94 (4.98-8.90)	8.66 (5.20-12.12)	0.366	4.15 (3.79-4.50)	3.50 (3.09-3.91)	0.024
Alcohol intake (drinks/week)	4.1 (2.7-5.6)	4.0 (1.0-7.0)	0.935	5.1 (4.0-6.2)	5.0 (3.9-6.2)	0.880	2.1 (1.7-2.5)	2.5 (1.9-3.1)	0.317
Sperm concentration (millions/ml)	75.7 (66.6-84.9)	56.2 (35.0-77.5)	0.069	95.5 (81.1-109.9)	75.8 (57.9-93.7)	0.093	86.5 (75.0-98.0)	55.8 (46.1-65.5)	<0.001
Total sperm count (millions)	266.6 (229.2-304.1)	158.3 (86.6-229.9)	0.011	388.4 (320.8-456.1)	268.5 (185.4-351.5)	0.029	284.7 (242.7-326.6)	205.1 (154.6-255.6)	0.020
Progressively motile sperm (%)	57.1 (54.4-59.8)	48.7 (41.8-55.6)	0.011	59.8 (56.0-63.5)	61.0 (55.9-66.1)	0.688	53.6 (49.9-57.4)	55.6 (50.3-60.8)	0.544
CB-153 (ng/ml)	2.42 (1.84-3.00)	2.47 (1.70-3.25)	0.934	0.15 (0.13-0.17)	0.11 (0.10-0.13)	0.011	0.27 (0.22-0.315)	0.29 (0.25-0.34)	0.462
PFOA (ng/ml)	4.84 (4.59-5.10)	4.79 (4.32-5.27)	0.862	5.19 (4.81-5.57)	5.36 (4.84-5.89)	0.598	1.91 (1.33-2.48)	1.60 (1.24-1.96)	0.447
PFOS (ng/ml)	51.65 (48.04-55.26)	52.78 (42.88-62.69)	0.800	12.12 (17.19-19.05)	19.33 (17.78-20.88)	0.160	8.20 (7.52-8.88)	7.89 (6.93-8.84)	0.587

Table 2. Results from the exploratory spline analysis and the linear regression analysis.
Presented as p-value (r^2 adjusted).

Spline analysis				
Population	PFOA		PFOS	
	Outliers included p (r^2 adjusted)	Outliers excluded p (r^2 adjusted)	Outliers included p (r^2 adjusted)	Outliers excluded p (r^2 adjusted)
Greenland	0.153 (0.066)	0.198 (0.072)	0.080 (0.080)	0.116 (0.081)
Poland	0.730 (0.053)	0.417 (0.017)	0.625 (0.031)	0.747 (0.009)
Ukraine	0.015 (0.045)	0.414 (-0.034)	0.668 (-0.031)	0.675 (-0.038)
Total	0.068 (0.020)	0.296 (0.036)	0.005 (0.054)	0.016 (0.057)
Linear regression analysis				
Greenland	0.976 (0.013)	0.943 (0.013)	0.941 (0.013)	0.762 (0.014)
Poland	0.696 (0.030)	0.417 (0.017)	0.625 (0.031)	0.747 (0.009)
Ukraine	0.017 (0.019)	0.414 (-0.034)	0.668 (-0.031)	0.675 (-0.038)
Total	0.050 (0.013)	0.845 (0.015)	0.026 (0.016)	0.118 (0.022)

Table 3. Results from the pairwise comparison of quartiles, outliers included, presented as mean Y:X chromosome ratio for each quartile and p-values for comparisons.

Greenland									
PFOA					PFOS				
Quartile	1st	2nd	3rd	4th	1st	2nd	3rd	4th	
Y:X ratio	0.514	0.507	0.519	0.510	0.510	0.515	0.515	0.506	
Quartile	1st	x	0.717	0.768	0.979	x	0.834	0.804	0.705
	2nd	0.717	x	0.070	0.992	0.834	x	1.000	0.042
	3rd	0.768	0.070	x	0.288	0.804	1.000	x	0.036
	4th	0.979	0.992	0.288	x	0.705	0.042	0.036	x
Poland									
PFOA					PFOS				
Quartile	1st	2nd	3rd	4th	1st	2nd	3rd	4th	
Y:X ratio	0.504	0.502	0.506	0.501	0.504	0.503	0.503	0.504	
Quartile	1st	x	0.995	0.994	0.965	x	0.999	1.000	1.000
	2nd	0.995	x	0.836	1.000	0.999	x	1.000	1.000
	3rd	0.994	0.836	x	0.687	1.000	1.000	x	1.000
	4th	0.965	1.000	0.687	x	1.000	1.000	1.000	x
Ukraine									
PFOA					PFOS				
Quartile	1st	2nd	3rd	4th	1st	2nd	3rd	4th	
Y:X ratio	0.509	0.507	0.507	0.507	0.506	0.510	0.508	0.506	
Quartile	1st	x	0.999	0.992	0.997	x	0.911	0.998	1.000
	2nd	0.999	x	1.000	1.000	0.911	x	0.996	0.889
	3rd	0.992	1.000	x	1.000	0.998	0.996	x	0.996
	4th	0.997	1.000	1.000	x	1.000	0.889	0.996	x
Total									
Quartile	1st	2nd	3rd	4th	1st	2nd	3rd	4th	
Y:X ratio	0.507	0.509	0.508	0.510	0.507	0.504	0.509	0.513	
Quartile	1st	x	0.991	0.999	0.853	x	0.690	0.999	0.242
	2nd	0.991	x	1.000	0.995	0.690	x	0.390	0.006
	3rd	0.999	1.000	x	0.979	0.999	0.390	x	0.433
	4th	0.853	0.995	0.979	x	0.242	0.006	0.433	x

10 **Table 4.** Trend analysis of categorized exposure data compared to Y:X chromosome ratio.
 11

Trend analysis of categorized exposure data												
Population	PFOA						PFOS					
	Outliers included			Outliers excluded			Outliers included			Outliers excluded		
	B	p	95 % CI for B	B	p	95 % CI for B	B	p	95 % CI for B	B	p	95 % CI for B
Greenland	0.000	0.912	-0.003 – 0.003	0.000	0.873	-0.003 – 0.003	-0.002	<i>0.028</i>	-0.004 – 0.000	-0.002	<i>0.044</i>	-0.004 – 0.000
Poland	0.000	0.710	-0.003 – 0.002	-0.001	0.603	-0.003 – 0.002	<0.001	0.983	-0.002 – 0.002	0.000	0.885	-0.002 – 0.002
Ukraine	-0.001	0.591	-0.003 – 0.002	-0.001	0.312	-0.004 – 0.001	0.000	0.840	-0.003 – 0.002	0.000	0.906	-0.003 – 0.002
Total	0.001	0.324	-0.001 – 0.003	0.001	0.548	-0.001 – 0.002	0.002	<i>0.017</i>	0.000 – 0.004	0.002	<i>0.039</i>	0.000 – 0.004

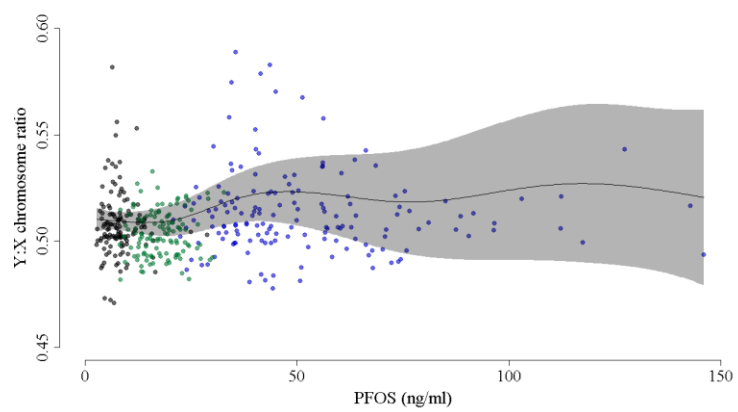
Figure 1

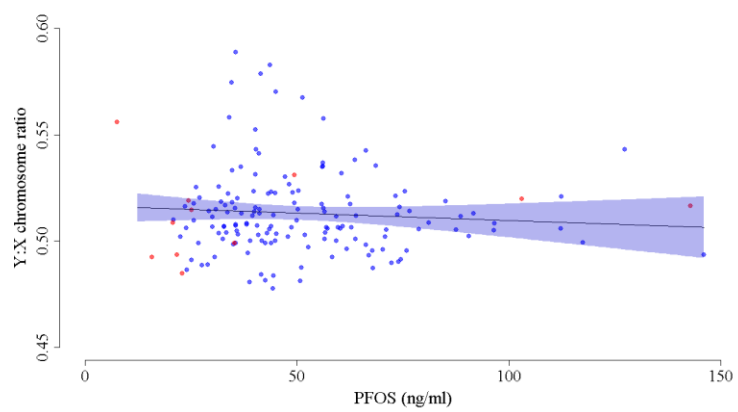
Figure 2 A

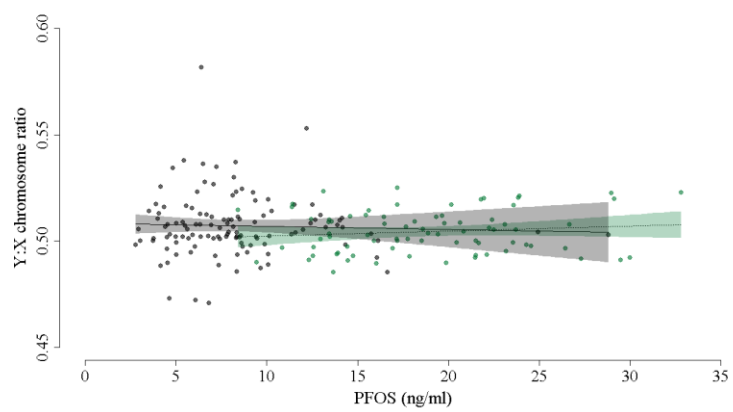
Figure 2 B

Figure 2 C