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Research Article

Exposure to Perfluoroalkyl Substances and Sperm DNA Global Methylation in Arctic and European Populations

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Perfluoroalkyl substances (PFASs) are widely used in a variety of industrial processes and products, and have been detected globally in humans and wildlife. PFASs are suspected to interfere with endocrine signaling and to adversely affect human reproductive health. The aim of the present study was to investigate the associations between exposure to PFASs and sperm global methylation levels in a population of non-occupationally exposed fertile men. Measurements of PFASs in serum from 262 partners of pregnant women from Greenland, Poland and Ukraine, were also carried out by liquid chromatography tandem mass spectrometry. Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorohexane sulfonic acid (PFHxS), and perfluorononanoic acid (PFNA) were detected in 97% of the blood samples. Two surrogate markers were used to assess DNA global methylation levels in semen samples from the same men: (a) average DNA methylation level in repetitive DNA sequences (Alu, LINE-1, Satα) quantified by PCR-pyrosequencing after bisulfite conversion; (b) flow cytometric immunodetection

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of 5-methyl-cytosines. After multivariate linear regression analysis, no major consistent associations between PFASs exposure and sperm DNA global methylation endpoints could be detected. However, since weak but statistically significant associations of different PFASs with DNA hypo- and hyper-methylation were found in some of the studied populations, effects of PFASs on sperm epigenetic processes cannot be completely excluded, and this issue warrants further investigation. Environ. Mol. Mutagen. 55:591–600, 2014.

Key words: perfluoroalkyl substances; human spermatozoa; DNA methylation; pyrosequencing; flow cytometry; epidemiology

INTRODUCTION

Perfluoroalkyl substances (PFASs) are a vast group of chemically and biologically inert fluorinated chemicals, made up of carbon chains in which all the hydrogens of the hydrocarbon backbones are substituted with fluorine atoms [EFSA, 2008; Buck et al., 2011]. Due to their resistance to biological degradation, they can accumulate in the environment and undergo biomagnification [Buck et al., 2011]. In the last decades, PFASs have been widely used in a variety of industrial processes and products. As a result, PFASs can be detected almost ubiquitously, e.g., in water, plants, various foods, animals including fish, birds, and mammals, as well as in humans [EFSA, 2008; Suja et al., 2009; Domingo, 2012]. An important group of PFASs are the perfluorinated organic surfactants, which include perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). While PFOS is generally found at higher concentrations than PFOA in humans and in the environment, both substances have been intensively studied from a toxicological perspective. The main route of human exposure to these substances is through dietary intake, in particular through contaminated fish and game, as supported by recent correlation studies between food intake and PFASs body burden [D'Hollander et al., 2010; Domingo, 2012]. Mammals cannot metabolize PFOS or PFOA and therefore they can only be eliminated by excretion, with half-lives of the two compounds in humans estimated at 4.8 and 3.5 years, respectively [Olsen et al., 2007]. Once ingested, PFASs can cross the placental barrier [Jensen et al., 2012] and both PFOS and PFOA have been detected in human breast milk [EFSA, 2008; Stahl et al., 2011]. PFASs have been detected in human populations all over the world, but with considerable geographical differences [Calafat et al., 2007; EFSA, 2008; Fromme et al., 2009; Roosens et al., 2010; Stahl et al., 2011; Domingo, 2012; Cornelis et al., 2012; Lindh et al., 2012]. Due to recent restrictions on their manufacture, environmental modeling predicts that PFASs exposure will gradually decline in temperate regions, but will increase in Arctic regions until 2030 [Armitage et al., 2009; Butt et al., 2010].

A number of in vitro and in vivo test systems reveal no evidence of genotoxicity associated with either PFOS or PFOA [Fernandez Freire et al., 2008; Eriksen et al., 2010; Lindeman et al., 2012]. However, HepG2 cells exposed to PFOA displayed evidence of DNA strand break induction and increased concentrations of reactive oxygen species and 8-hydroxydeoxyguanosine [Yao and Zhong, 2005]. Animal experiments showed that PFASs bind to blood proteins and accumulate primarily in the liver and then in other organs such as kidneys. They induce hepatotoxicity and immunotoxicity, alter lipid metabolism, and chronic exposure in rodents induces liver, testicular and mammary tumors [Stahl et al., 2011]. In addition, adverse reproductive and developmental effects have been described, although there was no evidence of teratogenicity. The main PFASs toxicity pathways are considered to be peroxisome proliferator-activated receptor alpha (PPARα) activation, plus epigenetic changes leading to carcinogenesis. Finally, in human epidemiological studies have provided clues into possible associations between PFASs exposures and human adverse effects such as interference with fatty acid and lipoprotein metabolism, alteration of hormone levels, thyroid diseases, and diabetes [Lau et al., 2007; Melzer et al., 2010; Steenland et al., 2010; Stahl et al., 2011].

In 2009, PFOS was added to Annex B of the Stockholm Convention on Persistent Organic Pollutants (POPs). The European Food Safety Authority (EFSA) recommends Tolerable Daily Intake (TDI) levels of 150 ng/kg b.w. per day for PFOS and 1.5 μg/kg b.w. per day for PFOA [EFSA, 2008]. Globally there is a growing interest in the reproductive effects of PFASs in humans, both in occupationally exposed and general populations [reviewed in Stahl et al., 2011]. However, the results from epidemiological studies are often inconsistent and the observed effects on classical reproductive endpoints, if any, are weak.

Associations between serum concentrations of PFASs, biomarkers of semen quality (such as sperm morphology, motility or chromatin integrity) and serum reproductive hormone levels have been investigated in the present [Specht et al., 2012; Toft et al., 2012] and in other cohorts of healthy men [Joensen et al., 2009, 2013; Raymer et al., 2012]. In general, while there was no consistent evidence that PFASs exposure interferes with sperm integrity, an impact of high PFOS concentrations on sperm morphology [Joensen et al., 2009; Toft et al., 2012], as well as on testosterone [Joensen et al., 2013] and sex hormone binding globulin [Specht et al., 2012] levels were reported. Semen quality was recently shown

to be affected after in utero exposure to PFOA but not to PFOS [Vested et al., 2013].

DNA methylation is an epigenetic covalent modification that adds a methyl group to the C5 position of Cytosine within CpG dinucleotides, forming 5-methyl Cytosine (5-mC). DNA methylation is implicated in many cellular and developmental processes such as embryonic reprogramming, cellular differentiation, imprinting, X chromosome inactivation, genomic stability, and complex diseases such as infertility and cancer [Carrell, 2012; Seisenberger et al., 2013]. There is increasing evidence that epigenetic mechanisms, including DNA methylation, may be influenced by environmental factors [Alegria-Torres et al., 2011; Feil and Fraga, 2012; Hou et al., 2012]. For example, global DNA methylation levels in peripheral blood lymphocytes (PBLs) have been found to be inversely associated with blood plasma levels for several Persistent Organic Pollutants (POPs) in a Greenlandic Inuit population [Rusiecki et al., 2008], while exposures to even low doses of POPs have been similarly associated with global DNA hypomethylation in a healthy adult population [Kim et al., 2010]. Associations between exposure to PFOA or PFOS and alteration of methylation levels at either specific loci or genome-wide have been described in a few in vitro and in vivo studies [Wan et al., 2010; Tian et al., 2012]. Furthermore, an epidemiological study reported an inverse dose response relationship between PFOA (but not PFOS) levels in umbilical cord blood from 30 newborns and global DNA methylation [Guerrero-Preston et al., 2010].

The aim of the present study was to evaluate the possible association between environmental exposure to PFASs and sperm DNA methylation among fertile men from Greenland, Poland and Ukraine.

MATERIALS AND METHODS

Study Population

The present study is part of a larger European study on fertility (www.inuendo.dk/clear) adopting a uniform protocol for data collection in Greenland, Kharkiv in Ukraine and Warsaw in Poland [Toft et al., 2005]. The study population was selected from a previously established cohort, consisting of 607 male partners of pregnant women [Bonde et al., 2008] of the project "INUENDO—Biopersistent organochlorines in diet and human fertility. Epidemiological studies of time to pregnancy and semen quality in Inuit and European populations" (http://www.inuendo.dk). The men selected in the present study completed a questionnaire on lifestyle, occupation, and reproductive history. Information about smoking habits and diet (seafood intake, caffeinated drinks, and alcohol consumption) was obtained with reference to the period when the couple tried to become pregnant. The questionnaires were translated into the native language and then back to English in order to be corrected from errors occurring during the first translation process. All the men included were at least 18 years old at the time of enrolment and signed an informed consent. The study was approved by local ethical committees collectively representing all participating populations.

Collection of Semen and Blood Samples

Semen and blood samples were collected between May 2002 and February 2004 as described by Toft et al. [2005]. Participants were instructed to collect semen samples after at least two days of sexual abstinence, and the duration of this abstinence was recorded. The collected sample was kept in contact with the body to maintain a temperature close to 37°C and immediately transported to the laboratory. Sperm concentration, motility, and morphology were analyzed according to a manual specifically defined for the project based on the latest version of the World Health Organization [WHO, 1999] guidelines [Toft et al., 2006]. Two snapcup cryotubes (VWR International, Roskilde, Denmark) with 0.2 mL aliquots of undiluted raw semen, collected 30 min after liquefaction, were prepared from each semen sample, coded and directly transferred into a −80°C freezer. For the purposes of the present study, we identified samples containing at least 8×10^6 spermatozoa, the lower operative limit to carry out all the epigenetic analyses described below. Among samples with sufficient material, 112 samples from Greenland, 100 from Warsaw and 100 from Kharkiv were randomly selected and included in this study. Coded frozen semen samples were shipped on dry ice to the Laboratory of Toxicology (ENEA Casaccia Research Center, Rome, Italy).

Blood samples were drawn from a cubital vein into 10 mL vacuum tubes for serum collection without additives (Becton Dickinson, Maylan, France). Serum was transferred with ethanol-rinsed Pasteur pipettes to ethanol-rinsed brown glass bottles (Termometerfabriken, Gothenburgh, Sweden). A piece of aluminum foil was placed on top of the bottles, which were then sealed. All sampling devices were prepared and sent from Lund University, Sweden, to Greenland, Poland and Ukraine. Venous blood samples were collected within 1 week of semen collection, except for some of the Greenlandic blood samples, which were collected up to 1 year in advance. The blood samples were centrifuged immediately after collection and sera were stored at −80°C for later analysis.

Sperm DNA Global Methylation Analysis

We used methylation levels of Alu elements and LINE-1 loci, determined by bisulfite treatment and PCR-pyrosequencing, as a proxy for sperm global methylation levels [Yang et al., 2004; Tost and Gut, 2007]. In order to increase the fraction of genomic coverage, we also analyzed methylation states of Satα repeats. These are non-transposonic repetitive satellite DNA sequences generally found in centromeres or centromere-adjacent heterochromatin that form the main DNA component of every human centromere. In addition to analyzing methylation levels of repetitive elements as surrogate markers of genome-wide methylation levels, sperm global methylation was also assessed on a cell-by-cell basis by flow cytometric (FCM) immunodetection of 5-mCs [Benchaib et al., 2005; Barzideh et al., 2013].

Alu, LINE-1, and Satα Methylation Assays by Pyrosequencing

DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), followed by treatment of 500 ng of DNA with the EpiTect Bisulfite kit (Qiagen), according to the manufacturer's protocol. Bisulfite-treated DNA was stored at −80°C and used shortly after treatment. Bisulfite-treated DNA (50 ng) was amplified in a 50 μL reaction mixture containing 25 μL of PyroMark PCR master Mix (Qiagen) and 0.4 μM of each primer. The analyses of Alu and Satα elements were conducted according to previously published methods [Yang et al., 2004; Bollati et al., 2007, 2009]. PCR conditions consisted of 96°C for 90 s, followed by 45°C for 60 s and 72°C for 120 s (45 cycles) for Alu; 95°C for 60 s, followed by 55°C for 60 s and 72°C for 60 s (45 cycles) for Satα. Primers sequences and fragment lengths are listed in Supporting Information Table I. LINE-1 methylation level was evaluated with PyroMark CpG LINE-1 kit (Qiagen). Pyrosequencing was carried out

using the PyroMark ID Q96 system (Biotage AB, Uppsala, Sweden) according to manufacturer's protocols. Controls were included in every pyrosequencing run to ensure the completion of bisulfite modification, the specificity of PCR amplification, and the success of pyrosequencing reactions. A Universal Methylated DNA standard (Zymo Research, Irvine, CA) was used as a control for bisulphite conversion. As controls for PCR and pyrosequencing reactions we used the EpiTect Control DNA (Qiagen), which includes methylated and unmethylated bisulfite-converted human control DNA. To ensure that there was no contamination, the negative controls of the PCR reactions in which DNA was replaced by water, also underwent pyrosequencing. Quantification of methylation levels was performed using the software provided. The percentage of methylation (%5-mC) was expressed for each DNA locus as the number of 5-mC divided by the sum of methylated and unmethylated cytosines. The Alu, LINE-1, and Satα methylation levels were measured at 4 consecutive CpG sites. From the quantitative results obtained by pyrosequencing, the average level of methylation for the whole fragment was calculated. All pyrosequencing analyses were performed on coded samples by a single investigator who was blind to the sample information.

LINE-1 analysis was conducted on 220 samples: 89 from Greenland, 64 from Warsaw (Poland) and 67 from the Kharkiv district (Ukraine). Satα analysis was conducted on 209 samples: 80 from Greenland, 62 from Warsaw and 67 from the Kharkiv district. Alu element analysis was conducted on 212 samples: 83 from Greenland, 64 from Warsaw and 65 from the Kharkiv district.

Flow Cytometric (FCM) Immunodetection of 5-mC: The FCM Sperm DNA Global Methylation (DGML) Assay

On the day of analysis, the samples were thawed in a 37°C water bath. After thawing, 1 mL of Phosphate Buffered Saline (PBS) containing 1% (v/v) Bovine Serum Albumine (BSA, Sigma, St. Louis, MO) was added to each vial. For the FCM immunodetection of 5-mC, we adopted previously published methods [Benchaib et al., 2005] with some modification. Samples were centrifuged (IEC Multispeed, Thermo Scientific, Rodano, Italy) at 2,000*g* for 5 min at 4°C and fixed in Carnoy solution (Methanol:Acetic acid, 3:1, v/v) for 15 min at room temperature, washed in 2 mL PBS, and centrifuged for 5 min at 2,000*g* at 4°C. To relax the nuclear structure, the fixed cells were incubated in 0.5 mL of a solution containing 25 mM dithiothreitol (DTT, Sigma), 0.1 % trypsin (Invitrogen, S. Giuliano Milanese, Italy), 2M NaCl (Sigma), and 25 mM Tris (Sigma), in Saline Sodium Citrate (Sigma) 2× (pH 7.2) for 15 min at room temperature. The cells were washed in 2 mL of 2× SSC, and centrifuged for 5 min at 2,000*g* at 4°C. DNA was denatured by adding 0.5 mL of 6 N HCl to the pellet for 15 min at room temperature, followed by neutralization with 3 mL of 0.1 M sodium tetraborate solution (Borax, Sigma). After centrifugation for 5 min at 2,000*g* at 4°C, the resulting pellet was washed twice in 2 mL of PBS, centrifuged for 5 min at 2,000*g* at 4°C, and finally resuspended in 1 mL PBS. Cell concentration was then measured in order to perform the immunostaining procedure with a known number of cells (1×10^6 cells). The immunoprobe consisted of a mouse IgG1 monoclonal antibody anti-5-Methylcytidine (5-mC) (Serotec, Clone 33D3, Oxford, UK) as the primary antibody, coupled with AlexaFluor488-conjugated Fab anti-mouse IgG1 fragments. The fluorescently (AlexaFluor488)-conjugated antibody complex was formed using Zenon® technology (Invitrogen) following the manufacturer's instructions. Spermatozoa were incubated in the dark with 100 μL of the fluorescently-labeled antibody complex at a concentration of 10 μg/mL in PBS for 60 min at room temperature. Samples were counterstained by adding 400 μL of a 5 μg/mL Propidium Iodide (PI, Sigma) solution and kept at room temperature in the dark for at least 10 min before FCM analysis.

Cells were analysed by a FACScalibur (Becton Dickinson, San José, CA) equipped with an air-cooled argon ion laser (488 nm) and standard optical filters to collect green (log scale) and red (linear scale) fluorescence. PI fluorescence intensity was used to identify true haploid cells, gating out debris and aggregates. Measurements stopped when a total of 10,000 events had been accumulated for each sample. Off-line analysis of the flow cytometric data was carried out by the FlowJo software (Tree Star, Inc., Ashland, OR). The mean, median, and standard deviation of the resulting green fluorescence intensity distribution were calculated. Values are expressed in channel number of the fluorescence acquisition scale (1,024 channels, 10-bit resolution).

Calibrite™ beads (Becton Dickinson) were used to ensure day-by-day flow cytometer standardization and stability. To check immunolabeling protocol variability, a reference semen sample from our laboratory repository was used. Aliquots of this reference sample were thawed and measured at the beginning and at the end of each FCM measurement session to establish repeatability of measurements. Inter-day variability of the methodology was 14.6%, evaluated after 11 flow sessions as the coefficients of variation (CV) of the average values of the mean of the green fluorescence distributions. The negative control was an aliquot of the reference sample incubated with AlexaFluor488-conjugated Fab anti-mouse IgG1 fragments alone, without the mouse monoclonal anti-5-mC IgG1 primary antibody. Immunostaining was confirmed by the visualization of the immunofluorescence in the head of sperm with an epifluorescence microscope (Carl Zeiss, Oberkochen, Germany). All FCM analyses were performed blind by two investigators.

A total of 262 samples were analyzed: 71 from Greenland, 96 from Warsaw (Poland) and 95 from the Kharkiv district (Ukraine). The discrepancies between the numbers of samples received and those successfully analyzed is due to random unpredictable cell loss occurring during the processing of some samples.

Analysis of PFASs

Blood samples were retrieved from a biobank and analysed for PFASs in 2011. The analysis of PFOS, PFOA, PFHxS, PFNA, perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA) and cotinine was performed by liquid chromatography tandem mass spectrometry (LC/MS/MS) [Lindh et al., 2012]. Labeled internal standards for all evaluated compounds were added to 0.1 mL aliquots of serum, and proteins were precipitated by addition of acetonitrile followed by vigorous shaking for 30 min. The samples were thereafter centrifuged and analyzed using an LC (UFLCXR, Shimadzu Corporation, Kyoto, Japan) connected to a hybrid triple quadrupole linear ion trap mass spectrometer equipped with a TurboIonSpray source (QTRAP 5500, Applied Biosystems, Foster City, CA). 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 as follows: PFOS 0.2 ng/mL; PFOA 0.6 ng/mL; PFHxS 0.06 ng/mL, PFNA 0.2 ng/mL, PFDA 0.2 ng/mL, PFUnDA 0.3 ng/mL, and PFDoDA 0.07 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 analyzed in triplicate across 3 different days, with final score calculated as the mean of the two closest measurements. Due to the high prevalence of samples below detection limits, PFDA, PFUnDA, and PFDoDA were excluded from analyses of correlation with sperm DNA global methylation levels.

Statistical Analysis

We calculated the mean ± SE of the measured PFASs, %5-mC of Alu, LINE-1 and Satα and the average channel number of the FCM DGML distributions. In addition, mean ± SE of the covariates age

TABLE I. Subject Characteristics, Sperm DNA Methylation Parameters, and PFASs Concentration both Combined and Divided into the Three Geographical Regions

Variable	Greenland	Kharkiv	Warsaw	Combined
Age (yrs)	30.8 ± 0.6	26.4 ± 0.5	30.2 ± 0.4	29.2 ± 0.3
	(*N* = 112)	(*N* = 97)	(*N* = 97)	(*N* = 306)
BMI (kg/m^2)	26.3 ± 0.4	24.5 ± 0.3	26.0 ± 0.3	25.6 ± 0.2
	(*N* = 110)	(*N* = 97)	(*N* = 96)	(*N* = 303)
Cotinine (ng/mL)	163.4 ± 15.0	156.7 ± 16.7	39.1 ± 9.5	121.6 ± 8.8
	(*N* = 112)	(*N* = 97)	(*N* = 98)	(*N* = 307)
Alcohol (drinks/week)	8.6 ± 1.1	2.8 ± 0.2	5.7 ± 0.6	6.1 ± 0.5
	(*N* = 110)	(*N* = 70)	(*N* = 88)	(*N* = 268)
Abstinence time (days)	3.8 ± 0.3	4.1 ± 0.2	6.8 ± 0.7	4.9 ± 0.3
	(*N* = 112)	(*N* = 97)	(*N* = 98)	(*N* = 307)
FCM DGML (channel no.)	238.2 ± 14.6	273.6 ± 13.9	240.9 ± 11.5	252.0 ± 7.7
	(*N* = 71)	(*N* = 95)	(*N* = 96)	(*N* = 262)
LINE-1, % 5-mC	79.1 ± 0.4	77.1 ± 0.9	75.5 ± 0.6	77.5 ± 0.4
	(*N* = 89)	(*N* = 67)	(*N* = 64)	(*N* = 220)
Alu, % 5-mC	20.8 ± 0.5	24.1 ± 0.4	23.3 ± 0.5	22.6 ± 0.3
	(*N* = 83)	(*N* = 65)	(*N* = 64)	(*N* = 212)
Satα, % 5-mC	51.1 ± 1.5	44.7 ± 1.6	42.3 ± 1.3	46.4 ± 0.9
	(*N* = 80)	(*N* = 67)	(*N* = 62)	(*N* = 209)
PFOS (ng/mL)	52.2 ± 2.5	7.9 ± 0.3	17.7 ± 0.5	27.2 ± 1.4
	(*N* = 112)	(*N* = 97)	(*N* = 98)	(*N* = 307)
PFOA (ng/mL)	5.0 ± 0.2	1.7 ± 0.2	5.3 ± 0.2	4.0 ± 0.1
	(*N* = 112)	(*N* = 97)	(*N* = 98)	(*N* = 307)
PFHxS (ng/mL)	3.0 ± 0.3	0.4 ± 0.1	1.2 ± 0.1	1.6 ± 0.1
	(*N* = 112)	(*N* = 97)	(*N* = 98)	(*N* = 307)
PFNA (ng/mL)	2.2 ± 0.2	1.1 ± 0.1	1.4 ± 0.1	1.6 ± 0.1
	(*N* = 112)	(*N* = 97)	(*N* = 98)	(*N* = 307)

Values represent the mean ± standard error.
BMI: body mass index; FCM DGML: flow cytometric sperm DNA global methylation level.

(years), body mass index (BMI; kg/m^2), cotinine (ng/mL), alcohol (drinks/week), and abstinence time (days) were calculated. General linear models (Proc GLM) were used to analyze adjusted associations between PFASs exposures (natural log transformed) and methylation markers. Methylation data were adjusted for age (years, natural log transformed) and smoking status (cotinine >10 ng/mL). The decision to adjust these data was made a priori, based on the available literature on potential associations between PBL global methylation and these covariates [Zhu et al., 2012].

Each of the three study populations (Greenland, Kharkiv and Warsaw) were analyzed independently, and also as one combined group with study population included as a covariate. For each of these four population groupings, serum levels of the four PFASs (PFOS, PFOA, PFNA, and PFHxS) were divided into tertiles. Spearman's correlation analysis was used to evaluate either crude associations between PFAS exposure and markers of sperm DNA global methylation, or the correlations among PFASs. Statistical analyses were performed using the SAS statistical software version 9.1 (SAS Institute, Inc., Cary, NC).

RESULTS

Demographic characteristics of the adult men enrolled from Greenland, Warsaw (Poland) and Kharkiv (Ukraine) are shown in Table I, together with the sperm DNA methylation end-points and the average values of blood levels of PFASs. For demographic measures, age was slightly lower in Kharkiv (average, 26.4 ± 0.5 years) than in Warsaw and Greenland (30.2 ± 0.4 and 30.8 ± 0.6 years, respectively). BMI was also slightly lower in Kharkiv (24.5 ± 0.3 kg/m^2) than in Warsaw and Greenland (26.0 ± 0.3 and 26.3 ± 0.4 kg/m^2, respectively). Cotinine level in Warsaw (39.1 ± 9.5 ng/mL) was lower than in Kharkiv (156.7 ± 16.7 ng/mL) and Greenland (163.4 ± 15.0 ng/mL). Alcohol consumption, expressed as number of drinks per week, was highest in Greenland (8.6 ± 1.1), followed by Warsaw (5.7 ± 0.6) and Kharkiv (2.8 ± 0.2). The lowest abstinence time (in days) was observed in Greenland (3.8 ± 0.3) followed by Kharkiv (4.1 ± 0.2) and Warsaw (6.8 ± 0.7).

For sperm DNA methylation endpoints, the average FCM DGML values (expressed as the channel number of the fluorescence intensity distribution ± SE) were 252 ± 7.7 for the combined study populations, with the highest value observed in Kharkiv and the lowest in the Greenland Inuit. Methylation levels measured after pyrosequencing (expressed as %5-mC ± SE), for LINE-1 revealed an overall mean of 77.5 ± 0.4, with the highest value reported in Greenland, followed by Kharkiv then Warsaw. For Alu, the overall mean was 22.6 ± 0.3, with the highest value observed in Kharkiv followed by Warsaw, and then Greenland. For Satα, we obtained an overall mean of 46.4 ± 0.9, with the highest value reported in Greenland, followed by Kharkiv and Warsaw.

Serum concentrations of the four considered PFASs differed among regions (Table I). PFOS occurred at the highest concentrations in all countries, at levels several-fold

higher than PFOA. PFHxS and PFNA occurred at the lowest concentrations, both exhibiting similar serum levels. The highest exposure levels to PFOS, PFHxS and PFNA were found in Greenland, whereas PFOA contamination was marginally higher in Warsaw. For all the considered exposures the lowest levels were found in Kharkiv. Serum levels of individual PFASs were all significantly correlated (Table II), with coefficients ranging from $r = 0.42$ (PFOA vs. PFNA, $P < 0.001$) to $r = 0.91$ (PFOS vs. PFHxS, $P < 0.001$). Correlations between PFASs have been described more in detail in Lindh et al. [2012].

PFOS and Sperm DNA Methylation Level

When considering data from all three populations together, no significant associations between DNA methylation and PFOS exposure were observed. A negative association emerged between PFOS and FCM DGML in Warsaw where, for each unit increase in ln transformed PFOS concentration, a decrease of 108.4 FCM DGML units (95% CI −191.5 to −25.2) was observed. In Kharkiv a positive association with Satα methylation was detected, so that for each unit increase in the ln transformed PFOS units there was an increase in Satα of 8.2% (95% CI 0.6% to 15.8%) (Table III).

TABLE II. Spearman's Correlations Between Serum Levels (ng/mL) of PFASs Measured in 307 Samples

	PFOS	PFOA	PFHxS	PFNA
PFOS	1	0.656	0.910	0.571
PFOA	0.656	1	0.646	0.417
PFHxS	0.910	0.646	1	0.556
PFNA	0.571	0.417	0.556	1

All the correlations are statistically significant ($P < 0.001$).

PFOA and Sperm DNA Methylation Level

None of the methylation parameters were associated with variation in PFOA exposure, when considering all three populations combined. A positive association with LINE-1 was detected in Kharkiv where, for each unit increase in ln transformed PFOA concentration, LINE-1 increased by 2.6% (95% CI 0.3% to 5.0%). No other statistically significant associations were observed (Table III).

PFHxS and Sperm DNA Methylation Level

As observed for the previous pollutants, no changes in all the methylation parameters were detected as a function of exposure for PFHxS when considering all populations combined. A negative association emerged between PFHxS and FCM DGML specifically in Greenland, where for each unit increase in ln transformed PFHxS concentration, FCM DGML decreased by 74.9 units (95% CI −132.8 to −17.1). No other associations were observed.

TABLE III. Associations Between PFASs Levels and Sperm DNA Global Methylation Parameters

	β-Coefficient* (CI 95%)			
PFAS	FCM DGML (Channel no.)	LINE-1 (% 5-mC)	Alu (% 5-mC)	Satα (% 5-mC)
PFOS				
Combined	−21.0 (−63.2; 21.3)	0.1 (−1.6; 1.9)	−0.4 (−1.7; 0.9)	1.1 (−3.1; 5.3)
Greenland	−32.1 (−105.6; 41.3)	−1.4 (−3.2; 0.4)	−0.1 (−2.2; 1.9)	−1.8 (−8.6; 5.1)
Kharkiv	27.2 (−43.1; 97.6)	2.5 (−1.9; 6.8)	−0.4 (−2.4; 1.6)	**8.2 (0.6; 15.8)**
Warsaw	**−108.4 (−191.5; −25.2)**	−0.6 (−4.9; 3.6)	−0.9 (−4.4; 2.6)	−7.2 (−16.0; 1.6)
PFOA				
Combined	−29.8 (−62.5; 2.9)	1.1 (−0.3; 2.5)	−0.3 (−1.5; 1.0)	0.6 (−3.3; 4.6)
Greenland	−37.5 (−126.5; 51.5)	−1.7 (−4.2; 0.7)	−0.3 (−3.2; 2.5)	−2.8 (−11.7; 6.1)
Kharkiv	−18.6 (−65.9; 28.6)	**2.6 (0.3; 5.0)**	0.1 (−1.4; 1.7)	2.8 (−3.3; 8.8)
Warsaw	−48.2 (−106.1; 9.7)	1.7 (−1.4; 4.8)	−0.9 (−3.5; 1.6)	−1.2 (−7.9; 5.4)
PFHxS				
Combined	−14.9 (−50.1; 20.3)	1.1 (−0.3; 2.6)	−0.3 (−1.4; 0.8)	1.5 (−2.0; 5.1)
Greenland	**−74.9 (−132.8; −17.1)**	0.1 (−1.3; 1.6)	−0.5 (−2.2; 1.2)	−0.1 (−5.3; 5.6)
Kharkiv	37.0 (−21.5; 95.6)	3.3 (−0.5; 7.2)	−0.1 (−1.9; 1.7)	4.0 (−3.0; 10.9)
Warsaw	−24.5 (−100.7; 51.6)	1.1 (−3.0; 5.3)	−0.2 (−3.7; 3.2)	−0.6 (−9.4; 8.2)
PFNA				
Combined	**−38.7 (−72.8; −4.6)**	1.1 (−0.3; 2.5)	−0.7 (−1.8; 0.3)	1.7 (−1.6; 5.1)
Greenland	−34.9 (−90.7; 20.9)	−0.1 (−1.4; 1.2)	−0.9 (−2.3; 0.6)	0.0 (−5.0; 5.0)
Kharkiv	20.2 (−50.4; 90.9)	**5.7 (1.4; 10.1)**	−0.8 (−2.9; 1.2)	**9.3 (1.5; 17.1)**
Warsaw	**−99.6 (−152.5; −46.8)**	0.2 (−2.5; 3.0)	−0.9 (−2.3; 0.6)	−1.22 (−7.1; 4.7)

Statistically significant ($P < 0.05$) associations are evidenced in bold.

All associations shown are mutually adjusted for site, age (ln transformed), smoking (yes/no).

*Regression coefficient representing the change (units of the DNA methylation variables per unit of ln transformed PFAS concentration).

PFNA and Sperm DNA Methylation Level

A negative association between PFNA and sperm DNA methylation measured by FCM DGML was observed for all three populations combined, with a FCM DGLM decrease of 38.7 units (95% CI −72.8 to −4.6) for each unit increase in ln transformed PFNA concentration. However, when analyzing the populations separately, this association only remained significant in Warsaw [−99.6 (95% CI −152.5 to −46.8). Interaction tests were suggestive of different associations in separate study populations. When measuring DNA methylation by bisulphite sequence analysis of repetitive DNA, the Kharkiv population showed evidence of positive associations between PFNA exposure and methylation states at both LINE-1 and Satα repeats. Specifically, LINE-1 methylation increased by 5.7% (95% CI 1.4% to 10.1%), with Satα methylation increased by 9.3% (95% CI 1.5% to 17.1%) for each unit increase in ln transformed PFNA concentration.

Presentation of Crude Association

In Supporting Information Tables II to V, median values (with the 25th and 75th percentiles) and crude Spearman's correlations of DNA methylation markers are reported as a function of PFASs exposure tertiles. The combined analysis across populations showed several statistically significant associations in the crude analysis that were not confirmed in the adjusted multivariate linear regression analysis, in particular, all the significant associations emerging from the combined populations. By and large, the country specific crude analyses and the adjusted analyses showed similar results, except for a statistically significant negative association of FCM DGML and PFHxS in Greenland which emerged in the adjusted but not in the crude analysis. Therefore, the crude relationships that survived the adjustment were: (i) PFOS and PFNA exposures significantly negatively associated with FCM DGML in Warsaw; (ii) PFOA and PFNA exposures positively associated with LINE-1 methylation level in Kharkiv; and (iii) a statistically significant positive association between PFNA concentration and Satα methylation level in Kharkiv.

DISCUSSION

No strong, consistent associations were detected between internal PFASs concentrations and any of the methylation biomarkers. Considering the population as a whole, PFOS, PFOA, and PFHxS were not associated with any of the global methylation outcomes, while PFNA was only associated with global methylation levels determined by FCM DGML. The crude analyses across populations showed several associations between PFASs exposure and sperm DNA global methylation markers that were not confirmed in the adjusted analyses. This is most likely due to differences in both exposure and methylation levels among the different geographical sites. Generally, statistically significant associations in one country could not be consistently observed in the other geographical areas. The sporadic associations of FCM DGML with exposure to PFASs pointed at a hypomethylation effect, whereas the sporadic association of LINE-1 and Satα pointed at hypermethylation effects. While differences in the target sequences and in the detection methods may certainly account for these inconsistencies, the overall data do not support a marked influence of PFASs exposure on sperm DNA methylation.

The hypothesis of a negative impact of environmental agents on the epigenetics of human somatic cells is being increasingly investigated [Szyf, 2011]. For example, LINE-1 and Alu methylation levels in PBLs are indeed modified by exposure to a variety of environmental stressors [reviewed in Alegria-Torres et al., 2011; Feil and Fraga, 2012; Hou et al., 2012]. However, to date very few studies have been conducted to evaluate effects of environmental agents on sperm DNA global methylation [Kumar et al., 2013].

To the best of our knowledge, the present study is the first evaluating human sperm DNA global methylation levels by both FCM immunodetection and pyrosequencing analysis of repetitive sequences in relation to PFASs body burden. Genetic variability, diet, lifestyle, or a combination of all these factors can determine the patterns of PFASs contamination in human populations [EFSA, 2008]. The average concentrations of PFASs in the present study are similar to those detected in a Danish study [Joensen et al., 2009], and slightly lower compared with those reported in an American survey [Raymer et al., 2012]. Greenland emerges as a polluted area with the highest contamination levels of PFOS, PFHxS and PFNA. A detailed description of exposure differences among our studied populations and determinants of exposure to PFASs can be found elsewhere [Lindh et al., 2012; Toft et al., 2012]. Differences in exposure levels or main determinants of exposure levels do not seem to explain the observed differences among populations in the associations between PFASs exposure and methylation level.

Sperm DNA global methylation level was recently shown to be a biomarker of fertility independent from the conventional semen quality parameters and sperm chromatin integrity [Benchaib et al., 2005; Aoki et al., 2006]. Sperm are the terminally differentiated male germ cells whose methylation pattern is progressively established during spermatogenesis [Carrell, 2012]. Most DNA methylation is acquired by the type A spermatogonial phase but several loci still undergo acquisition and loss of methylation marks from this stage to the pachytene spermatocyte phase, when patterns similar to those of mature

spermatozoa are observed [Chan and Trasler, 2011]. Both mRNAs and proteins of DNA methyltransferases DNMT1, DNMT3A, and 3B have been found throughout the human spermatogenic cycle [Marques et al., 2011].

Due to the long half-life of PFASs in humans [Olsen et al., 2007], one semen sample represents a good estimate of the exposure that occurred during at least one spermatogenic cycle, including exposure of the stem cells. Although all the men enrolled are spouses to pregnant women and thus 'fertile', it should be acknowledged that our population sample includes all degrees of subfertile men, including some men with low semen quality and/or long time to pregnancy.

The methylation levels of LINE-1 and Alu measured in our cohorts are similar to those reported in the literature [El Hajj et al., 2011; Kläver et al., 2012]. We note that pyrosequencing-based approaches, which provide a more precise assessment of possible methylation changes, could be misread as unmethylated mutated CpG sites that occur frequently in Alu sequences [Yang at al., 2004]. Furthermore, with the exception of weak associations between Satα and LINE-1 (Spearman's $r = 0.29$, $P < 0.0001$) and between Satα and Alu (Spearman's $r = -0.22$, $P = 0.002$), the various DGML end points are not intercorrelated in this study population. In agreement with our findings, it has been previously reported that environmentally-induced methylation changes at Alu and LINE-1 sequences do not necessarily behave at unison [Rusiecki et al., 2008; Baccarelli et al., 2009; Bollati et al., 2010; Kim et al., 2010]. The underlying mechanisms and significance of these alterations in DNA methylation are currently unknown.

In addition to differences in exposure levels to PFASs, other measures that differed between the three study populations were tobacco smoking, alcohol consumption, and abstinence time before sample collection. These factors are unlikely to have acted as confounding factors since none of them were associated with DGML in uni- and multivariate analyses [Consales et al., personal communication].

Data on repetitive sequence methylation levels in somatic and germ cells from the same individual are not available. However, it is worth noting that the LINE-1 and Alu methylation levels observed in our study are similar to those reported for PBLs and other somatic cells [Bollati et al., 2007; Rusiecki et al., 2008; Baccarelli et al., 2009; Pavanello et al., 2009; Pilsner et al., 2009; Tarantini et al., 2009; Bollati et al., 2010; Kim et al., 2010; Wright et al., 2010; Cash et al., 2011; Wernimont et al., 2011]. In contrast and as expected, the average Satα methylation level was lower than the level reported in somatic cells [Weisenberger et al., 2005; Yamagata et al., 2007; Bollati et al., 2009, 2011; Fabris et al., 2011; Molaro et al., 2011].

In conclusion, our results did not find any major consistent changes in sperm DNA global methylation after environmental exposure to PFASs. However, since weak but statistically significant associations between specific PFASs and methylation changes were found in some of the studied populations, effects of PFASs on sperm epigenetic processes cannot be completely excluded, and this issue warrants further investigation. More data on possible methylation changes at other loci and genomic regions, and mechanistic studies with experimental models are needed to thoroughly assess PFASs impact on male reproductive health.

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AUTHOR CONTRIBUTIONS

Drs. M.S., G.T. and J.P.E.B. contributed to the study design, data analysis and drafted the manuscript. Drs. I.O.S. and R.U. contributed to data analysis and manuscript drafting. Drs. G.L. and C.C. contributed to data collection and gave important intellectual input. Drs. P.E., T.M. and A.B. contributed to data collection. Drs. A.G., V. Z., J. K. L., H.S.P., D.H., C.H.L., and B.A.G.J. contributed to the study design, data collection and analysis. All authors revised the manuscript critically and approved the final version for publication.

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