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Perfluorooctanoic acid alters progesterone activity in human endometrial cells and induces reproductive alterations in young women

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ABSTRACT

Female fecundity is finely regulated by hormonal signaling, representing a potential target for endocrine-disrupting chemicals. Among the chemicals of most concern are the perfluoroalkyl substances (PFAS), widely used in consumer goods, that are associated with adverse effects on reproductive health. In this context, the endometrium clearly represents an important fertility determining factor. The aim of this study was to investigate PFAS interference on hormonal endometrial regulation. This study was performed within a screening protocol to evaluate reproductive health in high schools. We studied a cohort of 146 exposed females aged 18–21 from the Veneto region in Italy, one of the four areas worldwide heavily polluted with PFAS, and 1080 non-exposed controls. In experiments on Ishikawa cells included UV–Vis spectroscopy, microarray analysis and qPCR. We report a significant dysregulation of the genetic cascade leading to embryo implantation and endometrial receptivity. The most differentially-expressed genes upon PFOA coincubation were *ITGB8*, *KLF5*, *WNT11*, *SULT1E1*, *ALPPL2* and *G0S2* (all $p < 0.01$). By qPCR, we confirmed an antagonistic effect of PFOA on all these genes, which was reversed at higher progesterone levels. Molecular interference of PFOA on progesterone was confirmed by an increase in the intensity of absorption spectra at 250 nm in a dose-dependent manner, but not in the presence of β-estradiol. Age at menarche (+164 days, $p = 0.006$) and the frequency of girls with irregular periods (29.5% vs 21.5%, $p = 0.022$) were significantly higher in the exposed group. Our results are indicative of endocrine-disrupting activity of PFAS on progesterone-mediated endometrial function.

1. Introduction

Female fecundity is finely regulated by conserved genetic and epigenetic programs regulated by hormonal signaling pathways whose proper activation/inactivation leads to timely oocyte maturation and release, endometrial proliferation and embryo implantation. Diet, physical activity, stress, as well as exogenous agents such as endocrine-disrupting chemicals (EDCs) are known to alter this delicate balance potentially resulting in adverse health effects (Diamanti-Kandarakis et al., 2009). Among EDCs, perfluoroalkyl substances (PFAS) have recently become a class of great concern for public health because of their widely use in the manufacture of consumer goods and their presence in the soil and water (Lau et al., 2007). In particular, perflurooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) seem to be the two PFASs that are present in the highest concentrations in humans (Calafat et al., 2007; Starling et al., 2014). Recent epidemiological studies have documented a positive association between the presence of PFAS in human serum and perturbation of human reproduction (Reviewed in (Di Nisio and Foresta, 2019)). Yet, less is known about the association between PFOA and PFOS and female fertility.

Previous studies in mice have shown that animals who are exposed to PFOA experienced changes in ovarian function, timing of vaginal opening, and mammary tissue development (White et al., 2011; Yang et al., 2009; Zhao et al., 2012). Exposure to PFOA and PFOS have also been shown to increase neonatal mortality, neurotoxicity and immunotoxicity by altering foetus maturation and newborns development (Skakkebaek et al., 2001). Less clear, however, remains the effect of PFAS in humans. Some retrospective studies have found significant associations between PFAS exposure and reproductive hormone-

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sensitive outcomes including pubertal timing (Kristensen et al., 2013; Lopez-Espinosa et al., 2011), irregular cycles (Zhou et al., 2017), fecundity (Fei et al., 2009; Governini et al., 2011; Vélez et al., 2015), miscarriage (Darrow et al., 2014; Jensen et al., 2015), whereas other studies have not (Christensen et al., 2011; Louis et al., 2016; Vestergaard et al., 2012; Whitworth et al., 2012).

Given the potential toxicity due to PFAS on female reproductive health, studies to understand the potential pathological mechanism disrupting ovarian hormone pathways in women are timely and important. Embryo implantation represents the final and critical step in human reproduction. It is well known that the implantation process can be interrupted by progesterone receptor antagonists and selective progesterone receptor modulators (Fischer et al., 2012), which prevent pregnancy by inhibition or delay of ovulation, as well as direct effects on the endometrium (Baird et al., 2003; Lakha et al., 2007; Spitz, 2009). A recent study investigated the mechanism of action of PFOA on implantation by studying spheroid attachment on RL95-2 endometrial epithelial cells. The authors reported that PFOA (10–100 μM) significantly reduced spheroid attachment (Tsang et al., 2013), adding further evidence to the potential impact of PFAS on embryo attachment. In this context, the endometrium represents an important fertility determining factor (Strowitzki et al., 2006), even more sensitive to antiprogestins compounds than the hypothalamic–ovarian axis. Whether the endometrium represents a target of reproductive toxicity by PFAS has not been elucidated yet.

Here, we use human endometrial Ishikawa cells (Boehme et al., 2009; Hannan et al., 2010; Lessey et al., 1996; Naciff et al., 2010) to investigate the *in vitro* effect of PFAS interference on progesterone (P4) target genes by microarray analysis and RT-qPCR. *In vitro* studies were complemented with observational clinical studies using a population of young women from well-defined high-exposure areas reporting reproductive alterations, such as delayed puberty and menstrual cycles irregularity.

2. Methods

2.1. Ishikawa cell culture

Human endometrial epithelial adenocarcinoma Ishikawa cells (kind gift from prof. Marcello Maggiolini, University of Calabria, Italy) were cultured in minimum essential medium with Earle's salts and without phenol red (Invitrogen, Karlsruhe, Germany), supplemented with 2 mM L-glutamine, 1% non-essential amino acids and 5% charcoal-stripped FBS (all from Invitrogen) in 6 well tissue culture test plates at 37 °C under 5% CO_2. To study the effects of PFOA on P4-activated genes, cells were treated as described elsewhere for testing of *anti*-progestinic compounds (Fischer et al., 2012). Briefly, cells were seeded at a density of 50,000 cells/ml and grown for 3 days to subconfluency prior to addition of test substances. The medium of this priming phase was supplemented with 17β-estradiol (E2) 10^{-7} M (Sigma-Aldrich). Cells were subsequently co-incubated in triplicates for 48 h with test compounds applied with 0.1% ethanol or 0.1% DMSO as vehicle: P4 10^{-8} M (Sigma-Aldrich), alone or in combination with PFOA 10^{-6} M (Wellington Laboratories).

2.2. MTT assays

Cell proliferation was measured using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays. The cells were seeded in a 96-well culture plate at a density of 2×10^3 cells/well and incubated at 37 °C for 24 h. The cells were cultured in serum-free RPMI-1640 for 2 days, and then incubated with PFOA, P4 or a combination of both for an additional 48 h. A total of 20 μL of MTT (5 mg/mL in phosphate-buffered saline, PBS) was added to each well and the plates incubated in the dark at 37 °C for 4 h. Culture media was discarded and 200 μL of dimethyl sulfoxide (DMSO) added to dissolve the formazan crystals at room temperature for 10 min. The absorbance was measured at 490 nm using an iMark micro plate reader (BIO-RAD). Cell viability = OD_{490} nm of the treatment/OD_{490} nm of the control.

2.3. cDNA microarray analysis

To clarify the effects of PFOA on gene expression in endometrial cell line, we performed microarray analysis on three independent Ishikawa experiments. Total RNA was extracted from Ishikawa cell line by RNeasy Mini Kit (QIAGEN, Valencia, CA, USA). Dnase treatment was performed using Ambion® TURBO DNA-free™ Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instruction. RNA purity and concentration was assessed by NanoDrop ND-1000 (Thermo Fisher Scientific) and RNA integrity (RIN) was determined by 4200 Agilent Tapestation System using RNA ScreenTape (Agilent, Santa Clara, CA, USA). Synthesis and labelling of single-strand cDNA from 100 ng of total RNA was performed using WT Amplification Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) according to manufacturer's protocol. Successively, fragmented and labelled ss-cDNA was hybridized against the Affymetrix Human Clariom S Array (Thermo Fisher Scientific) for 17 h at 45 °C and 60 rpm. Arrays were subsequently washed and stained with streptavidin-phycoerythrin (GeneChip Wash and Stain Kit, Thermo Fisher Scientific) and scanned on an Affymetrix GeneChip Scanner 3000 7G scanner.

2.4. RT-qPCR analysis

Genes with high statistical significance ($p < 0.01$) from microarray experiment (*ITGB8*, *KLF5*, *WNT11*, *SULT1E1*, *ALPPL2* and *G0S2*) were confirmed by qPCR analysis. In addition to previous conditions, Ishikawa cells were also stimulated with PFOA alone, and with increasing concentrations of P4 (10^{-8} M, 10^{-7} M, 10^{-6} M) in order to exceed the binding of PFOA on the P4 in the culture medium. cDNA synthesis from total RNA (100 ng) was performed using SuperScript III (Invitrogen) and random hexamers. Pairs of primers targeting the selected and reference genes were designed using Primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCR primer sequences are provided in Table 1. Real Time-PCR was performed in a 20 μl final volume, containing 20 ng of cDNA, 1X Power SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) and a mix of forward and reverse primers (1 mmol/l each), on thermocycler StepOne plus (Applied Biosystems) using the following parameters: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s G6PDH was used as reference gene. Relative quantification was performed using Delta Delta Ct ($2^{-\Delta\Delta Ct}$) method.

Table 1
Primers used for qPCR analyses.

Gene	Forward	Reverse
SULT1E1	5′-GGAATGCAAAGGATGTGGCT-3′	5′-AGGAACCTGTCCTTGCATGA-3′
ALPPL2	5′-AAGCAGGAAAGTCAGTGGGA-3′	5′- GAGTACCAGTTGCGGTTCAC -3′
G0S2	5′-GAGAGGAGGAGAACGCTGAG-3′	5′-GAGGCGGGAATGACCTTAGT-3′
G6PD	5′-CATGAGCCAGATAGGCTGGA-3′	5′-ACAGGGAGGAGATGTGGTTG-3′
WNT11	5′-GATATCCGGCCTGTGAAGGA-3′	5′-CTTGTTGCACTGCCTGTCTT-3′
ITGB8	5′-ACAAGGATCAGCCTGTTTGC-3′	5′-ATGCCCAGCACACAGATTTC-3′
KLF5	5′-TTTGGAGAAACGACGCATCC-3′	5′-GTGAGTCCTCAGGTGAGCTT-3′

2.5. UV–vis spectroscopy

The UV–vis spectra were recorded on a Varian Cary50 spectrophotometer (Agilent Technologies, CA) with a data pitch of 1 nm and scan speed of 600 nm min−1. Quartz cuvettes of 1 cm were used. The absorbance measurements were performed at 25 °C in HBS (20 mM Hepes pH7.4, 0.145 M NaCl) containing 50% MeOH (V/V). Aliquots of PFOA (Wellington Laboratories, Ontario, Canada - 100 μg/ml, 247 mM dissolved in MeOH) were added to a progesterone or β-estradiol solution (Sigma-Aldrich, St. Louis, MO, USA - 10 μM).

2.6. Molecular docking simulation

Three dimensional molecular models of PFOA (cid: 9554), P4 (cid: 5994) and E2 (cid: 5757) were retrieved from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) as pdb files. The simulation of PFOA binding to P4 and E2 was then performed by using a docking procedure based on the Autodock Vina algorithm(Trott and Olson, 2010) implemented in the UCSF Chimera 1.12 (https://www.cgl.ucsf.edu/chimera/) molecular modelling software.

2.7. Subjects

This study was performed within the annual screening protocol to evaluate reproductive health in the high schools of Padova and surroundings (Veneto Region, Italy). The students are invited to fulfill an anonymous questionnaire to identify possible risk factors of the reproductive system in young adults. Based on the degree of pollution, local authorities (Veneto Region, 2016) have defined an highly-exposed area, the red area, which is the one with the highest PFAS levels, and in particular PFOA is the most representative chemical in this region. In utero exposure represents the most sensitive window to persistent endocrine disruptors and PFOA act on the foetus and newborns, leading to developmental defects (Skakkebaek et al., 2001), for this reason only life-long residents were considered for the study. Here, we report the findings of 1226 subjects who completed the questionnaire between June 2018 and March 2019. Subjects were then pooled in two groups on the basis of their residence: exposed (N = 146) and controls (N = 1080); response rates did not differ between the two groups (88.5% and 90.0%, respectively). For a subset of subjects from the two groups, serum levels of PFOA and PFOS were available within the regional screening protocol (Supplementary Table 1). The participants completed a questionnaire on sociodemographic characteristics, current and historic residential and employment information, lifestyle, and health variables. The questionnaire included two questions that was worded as follows: a) "At what age did you have your first period?" Girls were asked to digit the age (years). Girls who reported not to know/be sure about the beginning of the menstruation were excluded, b) "How do you define your periods?" Girls were asked to select one of the following categories: absent, irregular, mostly regular, regular. For statistical analysis, we generated a dichotomous variable: irregular cycles (absent or irregular) or regular cycles (always or mostly regular). The investigation was performed according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects, and the study was approved by the Research Ethics Committee of the University Hospital of Padova (N. 2208P). Participants did not receive any reimbursement.

2.8. Statistical analysis

The results were expressed as means ± SD or as medians + interquartile ranges. The Shapiro-Wilk W test for normality was used to check the distributions of the variables, non-normally distributed variables were log-transformed.; adjustment for multiple comparisons was calculated with the Bonferroni-Holm method. Differences between groups were analysed with Student's *t*-test. Linear regression analysis was performed for age at menarche, whereas binary logistic regression was performed for irregular menstrual periods. Covariates considered for inclusion in the models were demographic and lifestyle factors available in the data set as follows: age at survey, BMI, ethnicity, current smoking (yes or no), current alcohol intake (yes or no). Pearson's chi-square test, or Fisher's exact test when expected frequency was five or less, was used to examine the differences between groups for categorical variables. Spearman rank correlation coefficients were calculated to evaluate the correlations between continuous variables.

Microarray analyses were carried out using R statistical software packages. CEL files were processed using the 'affy' package and the resulting RMA (Robust Multi-array Average) (Irizarry et al., 2003) normalized data from each sample were merged into a single matrix. Probes' expression values were collapsed to genes using their maximum as the gene-wise value. After quality check on the normalized data, one of the three control replicates was excluded for subsequent analyses. In order to minimize biases, within each of the three subgroups and for each gene, possible outliers were identified, and excluded from the following analyses, by selecting the data points whose differences from the subgroup median were greater or equal to 95% of the difference between the subgroup minimum and maximum values. For each gene, within-subgroup and overall variances were calculated using the 'var' function of the 'stats' package. T-test nominal *p* values for the two hormone-treated subgroups were calculated using the 't.test' function of the 'stats' package. The expression signature was identified by the following procedure: 1) the third tertile of genes ranked by variance calculated over all samples was intersected with the genes selected within the first tertile of the within-subgroup variances for each subgroup, with the aim of enriching for genes showing at the same time the biggest variation among subgroups and the smaller variation within subgroups; 2) the above shortlist was then further intersected with the third tertile of genes showing the highest log2 ratio between the medians of the treatment subgroups. An unsupervised clustering was finally performed using the 'heatmap.plus' function from the heatmap.plus' package with the 'minkowski' distance method and 'ward.D2' clustering algorithm and visualized using the 'heatmap3' function from the 'heatmap3' package. KEGG pathways enrichment analysis was carried out using EGAN software (version 1.5) (Paquette and Tokuyasu, 2010), which calculates over-represented association nodes by standard one-tailed Fisher's exact test. *p*-values were calculated by means of the 't.test' function of the 'stats' package.

Data from qPCR experiments were compared by one-way ANOVA with Dunnett *post hoc* test to examine differences in gene expression between treatment groups and control. All statistics were calculated using SPSS (Version 23; SPSS Inc., Chicago, IL, USA). Two-sided *p* values < 0.05 were considered as statistically significant.

3. Results

Microarray analysis was performed in three independent Ishikawa experiments in order to identify P4-responsive genes that are dysregulated by co-incubation with PFOA. 173 genes resulted as differentially regulated in P4 + PFOA treatment compared to P4-treated cells (Fig. 1). By functional clustering, these genes were grouped in three main pathways: progesterone-mediated oocyte maturation, ErbB signaling pathway and oocyte meiosis. Out of these 173 genes, highly significant dysregulated genes with a *p*-value < 0.01 were selected for subsequent qPCR confirmation: *ITGB8*, *KLF5*, *WNT11*, *SULT1E1*, *ALPPL2* and *G0S2*, which are, along with computed logarithmic median fold change and p-values, listed on Table 2. All of these genes were significantly upregulated after P4 stimulation, compared with unstimulated controls (Fig. 2; all $p < 0.05$). By qPCR, we confirmed an antagonistic effect of

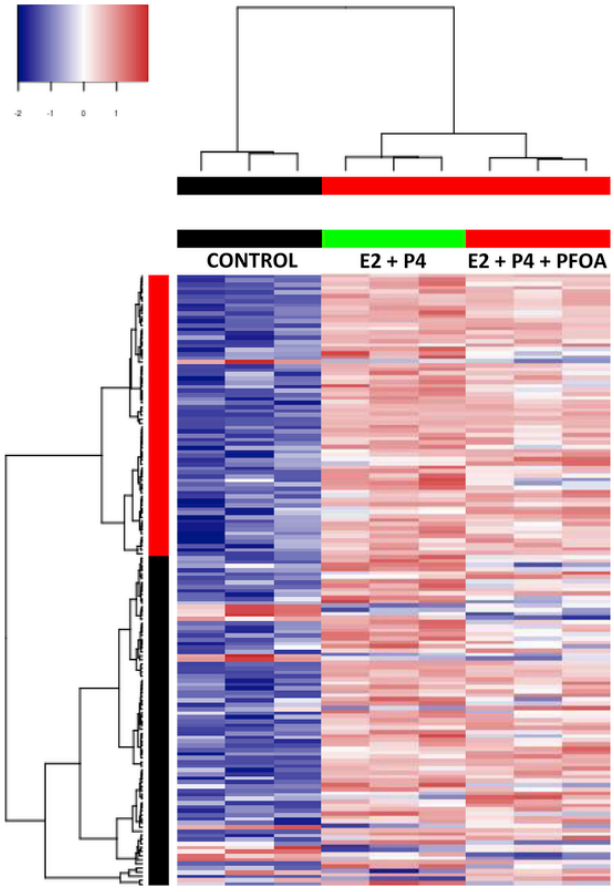

Fig. 1. Heat-map analysis of microarray data showing hierarchical clustering of differentially expressed probes between P4 and P4 + PFOA treated cells, after E2 priming, compared with untreated control. Each group has three replicates. Red or blue colors indicate differentially up or downregulated genes, respectively. The mean signals were background corrected and transformed to the log2 scale. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

PFOA 10^{-6} M on *ITGB8*, *KLF5*, *WNT11*, *SULT1E1*, *ALPPL2* and *G0S2* (Fig. 2; all $p < 0.05$). Stimulation with PFOA alone did not elicit any variation in gene expression, compared with basal condition (Fig. 2). By increasing P4 concentration, starting from 10^{-7} M, the antagonistic effect of PFOA was blunted in all genes considered (Fig. 3, all $p < 0.05$). Interestingly, by further increasing P4 concentration up to 10^{-6} M, all genes except *WNT11* were significantly further upregulated even in the presence of PFOA, compared with standard P4-only condition (10^{-8} M). Neither treatment (PFOA, P4, or a combination of both) did significantly affect Ishikawa cell proliferation compared with controls (all $p > 0.05$).

In order to understand PFOA antagonistic effect on E2-primed and P4-stimulated endometrial cells, PFOA interaction with the two hormones was evaluated. Data from docking simulations (Fig. 4A) suggested that PFOA can bind to P4 and E2, as expressed by their Gibbs free energy (ΔG) changes upon binding, although with less affinity to the latter than P4. To confirm, we tested directly the interaction between E2, P4 and PFOA by UV–Vis spectroscopy. Interestingly, PFOA occurred with an increase in the intensity of P4 absorption spectra at 250 nm in a dose-dependent manner, whereas in the presence of E2 we can only appreciate a significant increase at the wavelength of maximum absorbance (280 nm). In the range 200–320 nm PFOA does not absorb and cannot interfere with the analyte spectra. These evidences suggest that PFOA can directly interact with P4 (Fig. 4B).

Clinically-relevant parameters are reported on Table 3. After correction for multiple comparisons, age at menarche and the frequency of girls with irregular periods were significantly higher (+164 days) in the exposed group compared with control group (Table 3). The questionnaire provided some information on contraceptive pill usage from responses to an open question on contraceptive methods, which was low (10.7%) and did not differ between groups (10.6% in the control group and 11.0% in the exposed group, Table 3). All subjects were white Caucasian, except two girls in the control group and one in the exposed group. For this reason ethnicity was not further considered as confounder in subsequent analyses. PFOA, but not PFOS, serum levels were also consistently higher in the exposed group compared with controls (Supplementary Table 1). As age at menarche significantly correlated with age at survey and BMI, these factors were added as covariates in the regression analyses. By linear regression analysis, the main factors predicting age at menarche were: group ($p < 0.001$), BMI ($p < 0.001$) and age ($p = 0.011$). The difference between groups in the age at menarche remained statistically significant also after correction for BMI and age by ANOVA ($p < 0.001$). The prevalence of self-reported irregular cycles was significantly greater in the exposed group compared with controls (29.5% vs 21.5%, Table 3). By binary logistic regression analysis, irregular menstrual cycles were significantly predicted by group ($p = 0.025$), but not other covariates (age, BMI, smoking, alcohol), resulting in increased odds of having irregular cycles for exposed women (OR = 1.53, 95% CIs = 1.04–2.25).

4. Discussion

This study helps to establish a possible mechanism by which PFOA may affect female reproduction, and as a result, may inform future

Table 2
Gene transcripts most responsive to combined treatment with P4 and PFOA by microarray analyses and qPCR analyses.

Gene name	Gene symbol	Microarray		qPCR		Biological function
		$\log_2$ ratio [a]	p-value	$\log_2$ ratio [a]	*p*-value	
Wingless-Type MMTV Integration Site Family, Member 11	WNT11	0.033	0.001	1.936	0.035	Activation of cell proliferation
Kruppel Like Factor 5	KLF5	−0.024	0.001	−0.670	0.028	Transcription factor involved in endometrial receptivity
Integrin Subunit Beta 8	ITGB8	−0.044	0.008	−0.151	0.019	Adhesion protein involved in embryo attachment
Sulfotransferase family 1E, estrogen-preferring, member 1	SULT1E1	−0.086	0.002	−2.879	0.008	Inactivation of estrogens
Alkaline phosphatase placental-like 2	ALPPL2	−0.311	< 0.001	−0.874	0.015	Dephosphorylation of molecules
G0/G1 switch gene 2	G0S2	−0.029	0.004	−0.745	0.044	Cell cycle regulation

[a] $\log_2$ of the ratio between the medians of the treatment with P4 + PFOA *vs* P4 alone. [−] indicates down-regulation of the target genes.

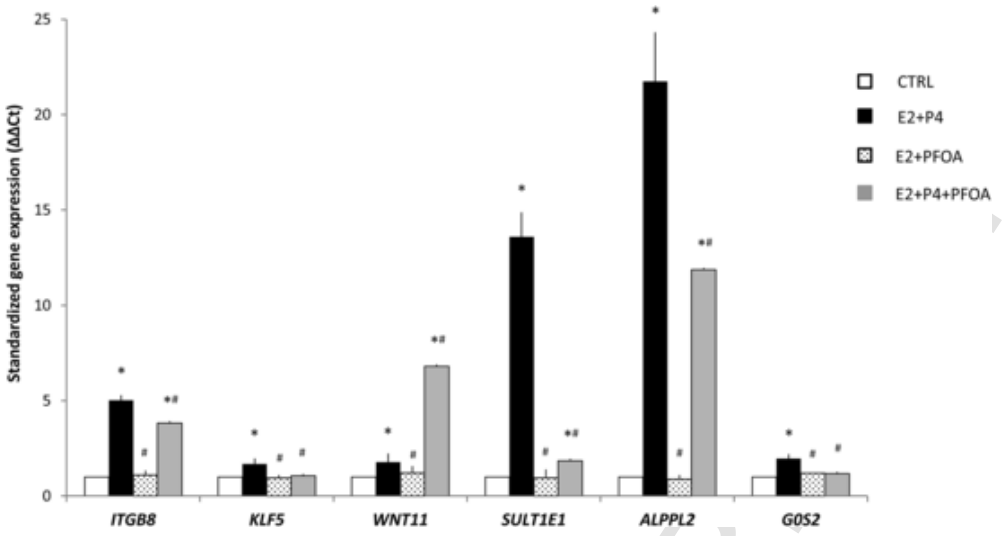

Fig. 2. qPCR analyses of *ITGB8*, *KLF5*, *WNT11*, *SULT1E1*, *ALPPL2* and *G0S2* gene expression in cultured human endometrial Ishikawa cells. Cells were pre-incubated with 17β-estradiol (E2) 10^{-7} M for 3 days and subsequently co-incubated in triplicates for 48 h with Progesterone (P4) 10^{-8} M (black bars), PFOA 10^{-6} M (dotted bars), or a combination of both (grey bars), compared with unstimulated controls (empty bars). Gene expression data are reported with the ΔΔCt method referred to *G6PDH* as the housekeeping gene and standardized to CTRL. Results are reported as means ± SD. * = $p < 0.05$ *vs* CTRL; # = $p < 0.05$ *vs* E2 + P4.

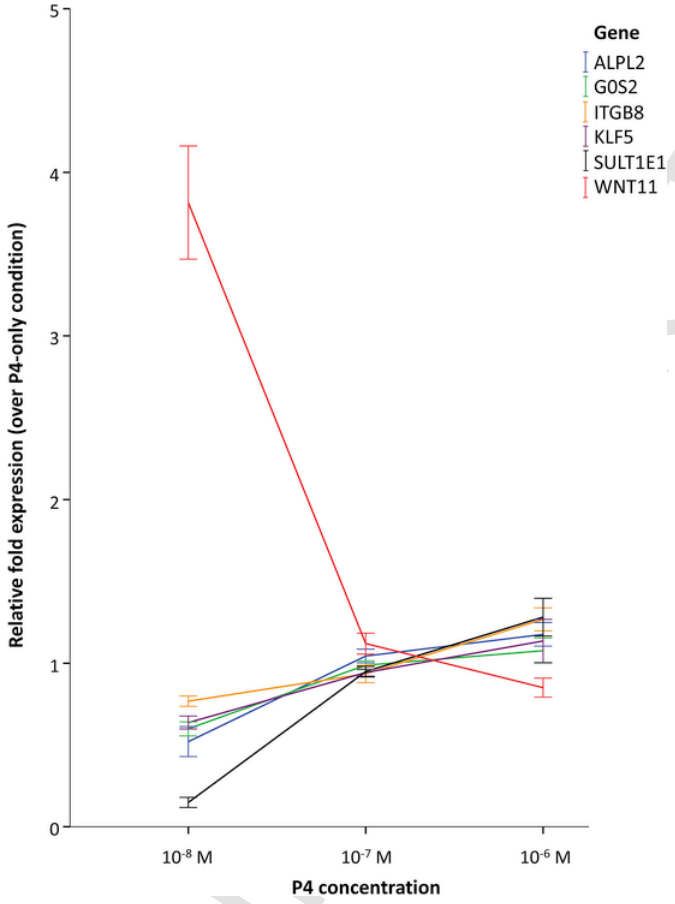

Fig. 3. Fold expression of *ITGB8*, *KLF5*, *WNT11*, *SULT1E1*, *ALPPL2* and *G0S2* genes in Ishikawa cells stimulated with PFOA 10^{-6} M in combination with P4 at increasing concentrations (10^{-8} M, 10^{-7} M, 10^{-6} M), relative to P4-only stimulation (10^{-8} M). Each gene is represented by a different color. Results are reported as means ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

research on PFOA in relation to hormone-sensitive women's reproductive health outcomes.

The human endometrium is unique among adult tissues, undergoing complex dynamic changes during each menstrual cycle under the control of E2 and P4. Understanding how ovarian hormonal pathways are impacted by PFOA is of primary importance given the role that E2 and P4 play in women's reproductive health and disease. P4 promotes the final differentiation of the endometrial lining and stimulates its secretory functions during the luteal phase to prepare the uterus for implantation (Strauss and Williams, 2004). Previous studies suggest that P4 production may also be impacted by PFOS and PFOA, disrupting ovarian function (Barrett et al., 2015). By computational modelling we observed a physical interaction between PFOA and P4 molecules, which was further supported by UV–vis spectroscopy experiments showing dose-dependent modification of P4 absorbance by PFOA. We selected the human endometrial Ishikawa cell line, which is one of the best-characterized human endometrial cell lines and expresses functional P4 receptor (Hannan et al., 2010; Lessey et al., 1996). We determined that PFOA treatment did not significantly affect Ishikawa cell proliferation. This result is consistent with those of previous studies, which showed that treatment of Ishikawa cells with lower concentrations of PFOA did not affect cell viability (Ma et al., 2016).

Based on our microarray and RT-qPCR results we identified six genes with highly significant dysregulation upon co-incubation of P4 with PFOA, whereas PFOA alone did not elicit any variation in gene expression, compared with basal conditions. In particular, the estrogen sulfotransferase (SULT1E1) in particular was the mostly inhibited genes upon PFOA stimulation, at concentrations reported in regions with point source drinking water contamination (1 μM) (Hölzer et al., 2008; Ingelido et al., 2018, 2010), compared with P4 alone. Remarkably, SULT1E1 was already indicated as the best target gene for evaluation of antiprogestin effects (Fischer et al., 2012) and plays a critical role in the inactivation of estrogens and in the pathogenesis of estrogen-dependent tumors (Pasqualini, 2009). Down-regulation of SULT1E1 by P4 antagonists may be of toxicological relevance since local estrogen levels could be increased by this mode of action, with possible effects on both menstruation and the hypothalamic-pituitary axis feedback, that could explain delayed puberty. By RT-qPCR analysis we demonstrated that the up-regulating effect of P4 on *ITGB8*, *KLF5*, *SULT1E1*, *ALPPL2* and *G0S2* mRNA levels was antagonized PFOA. In particular, *ITGB8*, *KLF5* and *ALPPL2* are involved in embryo

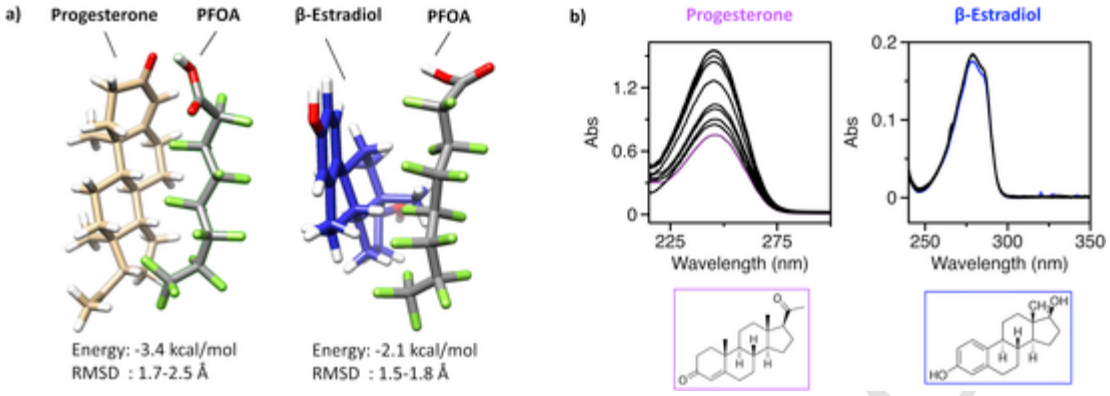

Fig. 4. (a) Docking simulation of PFOA (grey) binding to Progesterone (left, gold) and β-Estradiol (right, blue), with predicted Gibbs free energy changes upon binding and root mean square deviation (RMSD) of atomic positions. **(b)** (B) UV–vis spectra of progesterone and β -estradiol in the presence of increasing concentration of PFOA (0.5, 1, 1.8, 3, 5, 7, 9 mM). All the measurements were conducted at 25 °C in HBS/MeOH. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Characteristics of the study population stratified by PFAS exposure.

	All subjects (N = 1226)	Controls (N = 1080)	Exposed (N = 146)	*p*-value [a]
Age (years)	18.12 ± 0.76	18.12 ± 0.79	18.12 ± 0.56	1.0
Mother age at birth	32.04 ± 4.77	32.14 ± 4.81	31.26 ± 4.41	0.18
Weight (kg)	57.81 ± 9.42	57.62 ± 9.24	59.23 ± 10.64	0.21
Height (m)	1.66 ± 0.07	1.66 ± 0.07	1.66 ± 0.06	1.0
BMI (kg/m ^{2})	21.00 ± 3.14	20.94 ± 3.10	21.47 ± 3.46	0.21
Age at menarche (years)	12.32 ± 1.34	12.27 ± 1.34	12.72 ± 1.31	**0.006**
Smoking, n (%)	374 (30.5%)	322 (29.8%)	52 (35.6%)	0.09
Alcohol intake, n (%)	1166 (95.1%)	1025 (94.9%)	141 (97.2%)	0.15
Contraceptive pill, n (%)	131 (10.7%)	115 (10.6%)	16 (11.0%)	0.89
Irregular periods, n (%)	274 (22.3%)	231 (21.5%)	43 (29.5%)	**0.022**

[a] After adjustment for multiple comparisons. Chi-square test (nominal data) or Student's *t*-test (continuous data) was performed. Significant *p* values are in bold.

attachment, in agreement with reduced spheroid attachment induced by PFOA in experimental models (Tsang et al., 2013). Embryo implantation represents a critical step in embryo viability and growth, and it is well known that the implantation process can be interrupted by P4 antagonists (Fischer et al., 2012). Interestingly, most of these genes have already been reported as targets of antiprogestin chemicals in the same endometrial cell model (Fischer et al., 2012). Interestingly, WNT11 was the only gene upregulated by PFOA. It encodes for a protein involved in cell proliferation, which is typically stimulated in the proliferative phase of the endometrium upon E2 stimulation, and then downregulated in the progestin phase (Tepekoy et al., 2015), as mimicked by our experimental setting with a priming estrogenic phase and subsequent P4 stimulation. Interestingly, the expression pattern of all these genes was completely reversed by increasing P4 concentration up to 10^{-7} M in the presence of PFOA, upon expression levels comparable with cells stimulated with P4 10^{-6} M alone. Further increases in P4 levels up to 10^{-6} M resulted in even greater genes upregulation, with the exception of *WNT11*, which suggests that P4 is capable of reversing phenotype at higher concentrations, by exceeding the binding of PFOA on P4 in culture medium, leading to concentration-dependent gene regulation by P4, independently of PFOA. We hypothesize that the lack of downregulation of this gene could lead to a prolonged proliferative phase, and therefore cycle irregularities. Altogether these results support an antagonistic effect of PFOA on P4 target genes involved in implantation process and proliferation of endometrium, at least at physiological concentration, providing experimental evidence to the epidemiological association between PFAS exposure and longer time to pregnancy, miscarriage risk and subfecundity (Darrow et al., 2014; Fei et al., 2009; Governini et al., 2011; Jensen et al., 2015; Vélez et al., 2015).

Since menstruation has long been viewed as a proxy of female fecundity (Buck Louis et al., 2011; Harlow and Ephross, 1995), and dysfunction of menstrual cycle has been related to lower fecundity (Harlow and Ephross, 1995; Jensen et al., 1999; Mumford et al., 2012), our findings of an increased risk of irregular periods in young exposed women are in agreement with an hormonal interference of these chemicals on female fecundity, and confirm previous studies reporting increased odds of irregular menstrual cycles associated with PFAS exposure (Fei et al., 2009; Lyngsø et al., 2014; Zhou et al., 2017). Evidences from animal and human studies suggest that PFAS exposure results in alteration of menstrual periods (Barrett et al., 2015; Feng et al., 2015; Tsai et al., 2015). In animal studies, a 2-weeks exposure to PFOS (10 mg/kg) has been reported to cause persistent diestrus in rats (Austin et al., 2003). In mice, the chronic exposure to a low-dose PFOS (0:1 mg/kg/d) has also resulted in estrous cyclicity disruption in adult females (Feng et al., 2015).

Puberty is a vulnerable stage of life where disturbance has been linked to increased health problems. Thus, severe delays in the onset of puberty (>18 years) in girls have been reported to be a risk factor for infertility (Komura et al., 1992), and more modest pubertal delays (>13 years) have been associated with endometriosis, which may be in the pathway for infertility (Bérubé et al., 1998). Our results of an association between PFOA exposure and later age at having reached puberty (+164 days) are comparable with two previous studies reporting later menarche (+138 days and +159 day respectively) (Kristensen et al., 2013; Lopez-Espinosa et al., 2011). Our findings of statistically significant delays in the age of having reached puberty are of interest, although whether a later age in sexual maturation of 3–6 months has clinically important long-term impacts has yet to be determined.

Several endocrine disruptors have been reported to interfere with the timing of puberty, primarily in the direction of accelerated development (Crain et al., 2008). These associations could be due to be either central, with interference on the hypothalamic–pituitary axis, or peripheral, with direct effects on reproductive tissues or other target cells. Previous studies already provided evidence on the ovarian interference of PFOA and PFOS (Barrett et al., 2015; Chaparro-Ortega et al., 2018), by showing a reduction of steroid hormones (estradiol and progesterone) in both granulosa and theca cells. On this basis, we hypothesize that the alteration of puberty and menstrual cycles could be due either to the interference of PFOA on ovarian function or by

a direct effect of PFOA on P4 within endometrium, where the binding of PFOA on P4 could lead to an increased local concentration of this steroid, resulting in reduced or delayed drop of progesterone that normally induces the shedding of the uterine lining and therefore menstruation. In addition, in peripheral tissues, a variety of possible actions of endocrine disruptors include direct hormonal effect, modification of hormone receptor binding or alteration of the availability of naturally occurring hormones through disruption of synthesis, transport or metabolism (Caserta et al., 2007; Crain et al., 2008). Another mechanism by which PFOA exposure may influence pubertal timing is through its influence on concentrations of IGF-1 and thyroid hormones (Coperchini et al., 2017; Kim et al., 2018; Lopez-Espinosa et al., 2016). However, the underlying mechanism of PFAS on human reproductive system remains to be fully established.

Although our results allow no conclusion on the overall impact of PFOA on reproductive success, they identify these chemicals as being potentially hazardous to endometrial receptivity and embryo implantation, which are among the most critical processes in human reproduction.

The limitations of the present study include the small sample size of the exposed population and the lack of PFAS concentrations measures. However, we previously reported the concordance between geographic selection criteria and PFAS exposure pattern on a male population from the same area and of the same age (Di Nisio et al., 2019). Moreover, PFOA levels calculated on 13,856 subjects aged 14–40 years, during a surveillance program promoted by the Veneto region, reported consistent exposure in all subjects from this area (Veneto Region, 2018). Therefore, we believe that this population is representative of all those young women who live in the contaminated area in the Veneto region in Italy.

5. Conclusions

In summary, we present both experimental and epidemiological evidence supporting the endocrine-disrupting activity of PFOA on endometrial function. By using Ishikawa cell model, we report a significant dysregulation of the genetic cascade leading to embryo implantation and endometrial receptivity. Altogether our results are indicative of hormonal interference by PFOA, which is supported by a significant delay of puberty and increased odds of irregular periods in young women living in a highly-exposed area.

Authors contribution

A.D.N., M.S.R., I.S. and C.F. designed the study; M.D.R.P and C.C. evaluated questionnaires; A.D.N., I.S., M.S.R. and L.D.T. performed molecular experiments, L.A., N.P. and D.G. conducted analytical experiments; A.D.N., M.S.R. and C.Z. participated to data analyses; A.D.N., M.S.R. and C.F. wrote the manuscript and all authors approved the final version.

Declaration of competing interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.125208.

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