Endocrine Disruption of Androgenic Activity by Perfluoroalkyl Substances: Clinical and Experimental Evidence

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Background: Considerable attention has been paid to perfluoroalkyl compounds (PFCs) because of their worldwide presence in humans, wildlife, and environment. A wide variety of toxicological effects is well supported in animals, including testicular toxicity and male infertility. For these reasons, the understanding of epidemiological associations and of the molecular mechanisms involved in the endocrine-disrupting properties of PFCs on human reproductive health is a major concern.

Objective: To investigate the relationship between PFC exposure and male reproductive health.

Design: This study was performed within a screening protocol to evaluate male reproductive health in high schools.

Patients: This is a cross-sectional study on 212 exposed males from the Veneto region, one of the four areas worldwide heavily polluted with PFCs, and 171 nonexposed controls.

Main Outcome Measures: Anthropometrics, seminal parameters, and sex hormones were measured in young males from exposed areas compared with age-matched controls. We also performed biochemical studies in established experimental models.

Results: We found that increased levels of PFCs in plasma and seminal fluid positively correlate with circulating testosterone (T) and with a reduction of semen quality, testicular volume, penile length, and anogenital distance. Experimental evidence points toward an antagonistic action of perfluorooctanoic acid on the binding of T to androgen receptor (AR) in a gene reporter assay, a competition assay on an AR-coated surface plasmon resonance chip, and an AR nuclear translocation assay.

Discussion: This study documents that PFCs have a substantial impact on human health as they interfere with hormonal pathways, potentially leading to male infertility. ***(J Clin Endocrinol Metab* 104: 1259–1271, 2019)**

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Abbreviations: AGD, anogenital distance; AR, androgen receptor; ED, endocrine disruptor; ICC, interclass correlation coefficient; LC-MS, liquid chromatography coupled with high-resolution mass spectrometry; MMTV, mouse mammary tumor virus; MPFOA, marked perfluoroctanoic acid; MPFOS, marked perfluoroctanoic sulfonate; PFC, perfluoroalkyl compound; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; SPR, surface plasmon resonance; T, testosterone.

Perfluoroalkyl compounds (PFCs) are a class of organic molecules that are used in many everyday products such as oil and water repellents, coatings for cookware, carpets, and textiles. Their attractive physiochemical characteristics (*i.e.*, colorless, odorless, high thermal stability, low chemical reactivity and durability), high availability, and low cost ensure widespread use in the industry but also drive persistent accumulation into the environment, making them a potential biohazard for human health (1, 2). Indeed, PFCs have been found in human fluids and tissues, including the brain, placenta, and testis, which are protected by strong selective barriers (3–7). Interestingly, and for unknown reasons, there seems to be a sex-dependent pharmacodynamic profile, with adult men having a much higher tendency to have PFC accumulation and lower clearance (8–11).

Exposure pathways and toxicity mechanisms for PFCs are not well characterized, at least in humans [reviewed in Foresta *et al.* (12)]. An attractive hypothesis emerges from recent phenomenological studies correlating the dysfunction of the male reproductive system with the environmental levels of PFCs (13). PFCs may be absorbed by the intestine or inhaled, and once in the circulation, they may act as endocrine disruptors (EDs), ultimately leading to genital disorders, such as impaired spermatogenesis and reproductive defects, and antiandrogenic-driven conditions, such as testicular dysgenesis syndrome (13), which is an established risk factor for testis cancer (14, 15). PFCs could exert their toxicity on the fetus, newborn, and during development, especially in teenagers, due to alterations in sex hormone biosynthesis. Recent data suggest that *in utero* exposure to PFCs is associated with lower sperm quality and higher levels of LH and FSH at adulthood (16). Furthermore, by apparently acting as both antiandrogenic and antiestrogenic molecules, PFCs might also affect the downstream signaling pathways of sex hormones (17, 18), downregulate the hypothalamic-pituitary axis activity, and increase testicular toxicity during development (19–21).

The crucial emerging role of PFCs as pollutants of water, soil, and air and their persistent level in males warrant for more investigation on the mechanisms of PFC toxicity in humans. In this comprehensive study, we tested the hypothesis that human exposure to PFCs drives androgenic dysfunction and deterioration of the male reproductive system by altering the testosterone (T) interaction with its specific androgen receptor (AR). To investigate the relationship between PFC exposure and clinical alterations, we studied a cohort of 212 exposed young men from the Veneto region in the northeast of Italy. Along with the mid-Ohio valley in the United States, the Dordrecht area in the Netherlands, and the Shandong district in China, the Veneto region is one of the four areas worldwide heavily polluted with PFCs. To fully characterize the antiandrogenic action of PFCs and the structural and functional interaction between PFCs, AR, and T, we performed biochemical studies in established experimental models.

Methods

Subjects

This study was performed within the annual screening protocol to evaluate male reproductive health in the high schools of Padova and surroundings (Veneto region, northeast of Italy). The aim of this screening is to early diagnose possible risk factors and diseases of the male reproductive system. Here, we report the findings of 383 subjects who voluntarily agreed to complete the cross-sectional study between June 2017 and May 2018. Included subjects underwent an accurate medical visit, measure of anthropometric parameters, ultrasound examination of the testes, and semen analysis at our medical center. 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). The investigation was performed according to the principles of the Declaration of Helsinki. Participants did not receive any reimbursement. Based on geographical distribution of PFC pollution (22), subjects were then grouped on the basis of their residence. Regional authorities (23) have defined two different zones within the exposed area, based on the degree of pollution: the red area, which is the one with the highest PFC levels, and the yellow zone, with slightly lower levels, but at risk for close proximity to the contamination plume and surroundings (22). Among the 383 subjects included in the study, 83 were resident in the yellow zone, 129 in the red zone, and 171 outside the exposed area (green zone). Specific geographical origin is reported in the online repository (22). To increase the sample size for subsequent statistical analyses, subjects from the red and yellow zones were pooled together as a single exposed group, because no difference has emerged between the two areas for the clinical parameters considered (data not shown), except for nonprogressive sperm motility and immotile sperm that were mutually different between groups. Subjects from the green zone (nonexposed) were considered the control group.

Anthropometric measurements

Anthropometric and penile measurements included height, weight, body mass index, waist circumference, arm span, crown-to-pubis length, penile length, and circumference. These parameters are commonly used to suggest severe forms of congenital or prepubertal hypogonadism, such as patients with Klinefelter and Kallmann syndrome (24). Every measure was taken three times to the nearest millimeter. Height was accurately taken from the floor to the crown of the head as described in previous studies (25, 26). Body mass index was calculated using the formula weight (kg)/height (m)^2. Waist circumference was measured at the midpoint between the superior border of the iliac crest and the lowest rib (27). The arm span was measured as the distance between the tips of the middle fingers with the arms fully extended (28). The pubis-to-floor distance was measured from the upper edge of the pubic symphysis to the floor. The crown-to-pubis length was consequently derived as the difference between height and pubis-to-floor distance (29). The penile length was measured as the linear distance along the dorsal side of the penis extending from the lower edge of the pubic bone to the tip of the glans in the flaccid state. The penis circumference was measured at the middle of the shaft (30). All subjects were evaluated by the same two clinicians. The intraoperator variations were in all cases <5%. Testicular volumes were evaluated by ultrasound, using the standard ellipsoid formula (width × height × length × $\pi/6$, coefficient of variation <10%).

Anogenital distance

The anogenital distance (AGD) was measured as previously described elsewhere (31), from the posterior base of the scrotum to the center of the anus. The participant was placed in a supine, frog-legged position with his thighs at a 45° angle to the examination table. In a subset of 50 randomly chosen patients, AGD measures were repeated twice by the same technician and then blindly by the second examiner. Interclass correlation coefficients (ICCs) were calculated for repeatability estimation within and between examiners. Repeatability was very high within individuals (ICC = 0.979; 95% CI, 0.960 to 0.989) and slightly lower across examiners (ICC = 0.932; 95% CI, 0.873 to 0.964).

Semen collection and analysis

Human semen samples were obtained by masturbation after 2 to 7 days of sexual abstinence and stored in sterile containers. Samples were allowed to liquefy for 30 minutes at 37°C and were examined for seminal parameters according to World Health Organization criteria (32). Briefly, semen volume was measured by weighing, assuming a semen density of 1.0 g/mL; sperm concentration was evaluated by a hemocytometer (Bürker-Türk; Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany); sperm morphology was identified from semen smears prepared with 10 μL of well-mixed semen, stained with Papanicolaou, and assessed using the Tygerberg strict criteria. Sperm motility was graded into total (progressive + nonprogressive motility) and progressive motility. Total sperm count (volume × sperm concentration) was also calculated.

Seminal parameters were available for 211 exposed subjects and 170 controls because one subject in each group failed to collect semen.

Sex hormones quantification

Blood was collected in the fasting state between 08:00 and 10:00 AM. Serum total T, FSH, and LH were evaluated by commercial electrochemiluminescence immunoassay methods (Elecsys 2010; Roche Diagnostics, Mannheim, Germany). For all parameters, the intra- and interassay coefficients of variation were <8% and <10%, respectively. All determinations were performed in duplicate.

PFC quantification in serum and semen by mass spectrometry

In a subset of patients (50 controls and 50 exposed subjects), PFCs were evaluated in serum and seminal fluid. For serum analyses, cells are removed from plasma by centrifugation for 10 minutes at 2000 × *g*. Following centrifugation, the liquid component (plasma) was transferred into a clean polypropylene tube. The quantification of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) was processed on reversed-phase liquid chromatography coupled with high-resolution mass spectrometry (LC-MS) (Agilent Varian 320; Agilent Technologies, Santa Clara, CA). Briefly, each sample was dissolved in acetonitrile, and fixed amounts of the stable isotope-labeled internal standard were added (MPFOA [marked PFOA] and MPFOS, [marked PFOS]; Wellington Laboratories, Ontario, Canada). To test the analytical response and to optimize the calibration curve, a standard mixture was used at increasing concentrations (PFAC-MXB; Wellington Laboratories) together with isotope-labeled internal standards (MPFOA, MPFOS) at fixed concentrations. This solution was analyzed by LC-MS. The different perfluoroalkyl species were identified by comparing the retention time and mass spectra (*i.e.*, m/z value and isotopic pattern). Quantification of each species was calculated using the corresponding calibration curve.

AR gene reporter assay

All the transfections were performed in HeLa cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA) as previously described (33). Briefly, cells were cultivated in DMEM (ThermoFisher, Waltham, MA), supplemented with 5% fetal bovine serum (Sigma Aldrich, St. Louis, MO), antibiotics, and antimycotics in a humidified incubator at 37°C with 5% CO_2. Transient gene expression assay was performed in 96-well plates using Lipofectamine 2000 reagent (ThermoFisher) and a dual-luciferase reporter assay system (Promega, Madison, WI). HeLa cells were grown in 96-well plates and cotransfected at 70% confluence with 100 ng/well of the expression vector for the full-length human AR (pSV-AR0), 100 ng/well of mouse mammary tumor virus (MMTV)–luciferase reporter plasmid, and 10 ng/well of pGL4.74 *Renilla luciferase* (Promega) (internal control for transfection efficiency). pSV-AR0 and MMTV-Luc plasmids were a kind gift from Prof. Claessens (University of Leuven, Belgium). Twenty-four hours after transfection, media were replaced with fresh DMEM, and test chemicals (1 μM PFOA and PFOS; Wellington Laboratories) in the absence or presence of 10 nM T (positive control; Sigma Aldrich) were added to each well. Flutamide (1 μM, Sigma Aldrich) served as a negative control. Treated cells were harvested 24 hours later and lysed with lysis buffer of the dual-luciferase reporter assay (Promega). Luciferase activity was measured with a multilabel plate reader (Wallac Victor; Perkin-Elmer, Waltham, MA), and all data were standardized for luciferase activity. Results are shown as the mean ± SD of three independent experiments, each performed in duplicate.

Surface plasmon resonance analyses

Surface plasmon resonance (SPR) experiments were performed on a BIAcore-S200 instrument (GE-Healthcare, Chicago, IL) to monitor the interaction between T or PFOA and the AR. The binding domain AF2 of the AR (650-920; Abcam, Cambridge, MA) was covalently immobilized on a CM5 sensor chip using an amine-coupling chemistry. Binding experiments were carried out by injecting increasing concentrations of T (0 to 1 mM; Sigma Aldrich) and PFOA (0 to 4 μM; Wellington

Laboratories) at a flow rate of 30 μL/min, using 10 mM Hepes (pH 7.4), 0.15 M NaCl containing 3% MeOH (v/v) as running buffer. Each cycle consisted of a 60-second contact time, followed by 120-second dissociation and 30-second pulse with 100 mM Hepes (pH 7.4) as the regeneration step. The response units at the steady state were plotted as a function of [analyte], and the dissociation constant (K_d) was obtained as a fitting parameter of a binding isotherm. Competition experiments were performed to investigate the effect of PFOA on T-AR interaction. Solutions of T (250 μM) were incubated with different concentrations of PFOA (0 to 4 μM) for 10 minutes and then injected over the AR-coated sensor chip. All experiments were performed in triplicate at 25°C.

AR nuclear translocation assay

The clonal strain of the mouse MA-10 Leydig cell line used for the AR nuclear translocation assay was purchased from ATCC (CRL-3050). Cells were used at the second cell passage from original thawing to maintain the phenotype as close as possible to the one claimed by the manufacturer and handled as previously described (34). Briefly, cells were seeded on 0.1% gelatin-coated plasticware and maintained in DMEM/F12 medium, pH 7.7 (GIBCO-Invitrogen, Milano, Italy), supplemented with 20 mM Hepes, 15% horse serum, and 50 μg/mL gentamicin. Starved MA-10 cells were seeded onto glass slides (BD Biosciences, Milano, Italy) and cultured at different concentrations of T (1 to 100 nM; Sigma Aldrich) and PFOA (0.1 to 1 μM; Wellington Laboratories), alone or in combination. After 24 hours, cells were fixed with 4% paraformaldehyde/PBS solution for 15 minutes at room temperature and were permeabilized with 1% Triton X-100/PBS solution for 10 minutes at room temperature. Furthermore, samples were saturated with 5% BSA/5% normal donkey serum in PBS for 30 minutes and then incubated overnight at 4°C with rabbit polyclonal anti-AR antibody (ab74272; Abcam) for further assessment of AR nuclear translocation by means of relative quantification of fluorescence density. In the negative control, primary antibodies were omitted. The following day, primary immunoreaction was detected by incubation with IgG-FITC goat anti-rabbit secondary antibody (K1715; Santa Cruz Biotechnology, Dallas, TX). Finally, cells were counterstained with DAPI, mounted with antifade buffer, and analyzed with a videoconfocal fluorescence microscope (Nikon, Firenze, Italy). The nuclear translocation of AR (the intensity of the AR signal within the nucleus relative to the total intensity) was quantified in 20 to 40 cells using the ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analyses

All statistics were calculated using SPSS (version 23; SPSS, Inc., Chicago, IL). *P* values <0.05 were considered statistically significant. The results were expressed as means ± SDs or as medians (interquartile ranges). The Shapiro-Wilk *W* test for normality was used to check the distributions of the variables; as almost none of the parameters was normally distributed (except height, crown-to-pubis, pubis-to-floor, and sperm progressive motility), and almost all of log-transformed distributions did not satisfy normality, nonparametric statistics was applied. The Mann-Whitney test was used to assess differences between groups in the anthropometric, seminal, and hormonal parameters and in the concentrations of serum and seminal contaminants. Both raw and adjusted *P* values are

Table 1. Anthropometric and Seminal Parameters in 171 Controls and 212 Exposed Subjects

	Controls (n = 171)[a]			Exposed (n = 212)[a]				
Parameters	Mean ± SD	Minimum–Maximum	Median (IQR)	Mean ± SD	Minimum–Maximum	Median (IQR)	Raw *P*[b]	Adjusted *P*[c]
Age, y	18.7 ± 1.0	18.0–24.0	18.0 (18.0–19.0)	18.5 ± 0.8	18.0–22.0	18.0 (18.0–19.0)	0.081	0.567
Height, cm	179.2 ± 6.2	162.0–192.0	180.0 (175.0–184.0)	178.8 ± 6.9	160.0–203.0	179.0 (175.0–183.0)	0.575	1.0
Weight, kg	73.2 ± 8.5	54.0–96.0	73.0 (67.0–78.0)	73.9 ± 11.9	47.0–120.0	73.0 (65.0–80.0)	0.897	1.0
BMI, kg/m^2	22.8 ± 2.3	18.2–31.0	22.5 (21.1–24.0)	23.1 ± 3.1	16.6–35.8	22.5 (21.0–24.5)	0.492	1.0
WC, cm	81.8 ± 7.1	64.0–103.0	81.0 (77.0–85.6)	84.0 ± 10.5	63.5–140.0	82.0 (77.0–88.0)	0.174	0.87
Arm span, cm	182.1 ± 10.2	87.0–200.0	182.0 (178.0–187.6)	182.0 ± 8.2	160.0–204.0	181.5 (176.5–187.0)	0.276	1.0
Crown-to-pubis distance, cm	81.8 ± 4.9	70.0–94.0	82.0 (78.0–85.2)	82.9 ± 5.5	68.0–98.0	83.0 (79.0–86.0)	**0.041**	0.328
Pubis-to-floor distance, cm	97.4 ± 5.3	84.5–110.0	97.8 (93.0–101.2)	95.9 ± 5.7	79.0–117.0	96.0 (93.0–100.0)	**0.009**	0.09
Crown-to-pubis/pubis-to-floor ratio	0.8 ± 0.1	0.68–1.01	0.8 (0.8–0.9)	0.9 ± 0.1	0.6–1.2	0.9 (0.8–0.9)	**0.014**	0.126
Testicular volume, mL	16.1 ± 3.2	7.6–26.5	16.0 (14.1–18.0)	14.7 ± 3.2	6.8–24.5	14.5 (12.5–16.5)	**<0.001**	**<0.001**
Penis length, cm	9.7 ± 1.6	6.0–13.5	10.0 (8.5–11.0)	8.6 ± 1.7	2.0–13.5	9.0 (8.0–10.0)	**<0.001**	**<0.001**
Penis circumference, cm	10.0 ± 1.0	5.0–13.0	10.0 (9.5–10.5)	9.9 ± 1.1	7.0–13.0	10.0 (9.0–10.5)	0.134	0.804
AGD, cm	4.5 ± 0.8	2.5–7.2	4.5 (4.0–5.0)	4.1 ± 0.9	2.0–7.0	4.0 (3.5–4.5)	**<0.001**	**<0.001**
Semen volume, mL	2.7 ± 1.4	0.3–7.5	2.5 (1.5–3.5)	2.6 ± 1.3	0.2–7.0	2.5 (1.5–3.3)	0.512	0.568
pH	7.5 ± 0.2	7.0–8.0	7.5 (7.4–7.6)	7.6 ± 0.2	7.0–8.5	7.7 (7.5–7.7)	**<0.001**	**<0.001**
Sperm concentration, 10^6/mL	89.2 ± 97.9	0–800.0	65.0 (33.2–115.6)	66.2 ± 53.2	0–327.0	57.0 (25.3–99.0)	**0.045**	0.180
Total sperm count, 10^6	230.5 ± 292.6	0–2240	135.0 (66.0–281.1)	166.8 ± 154.5	0–817.5	123.0 (43.4–258.0)	**0.032**	0.160
Progressive motility, %	51.8 ± 15.5	0–91.0	53.0 (42.0–62.0)	44.1 ± 17.1	0–85.0	44.0 (32.0–57.0)	**<0.001**	**<0.001**
Nonprogressive motility, %	7.5 ± 6.5	0–32.0	6.0 (3.0–10.0)	8.2 ± 7.6	0–63.0	6.0 (4.0–10.0)	0.284	0.568
Immotile sperm, %	40.2 ± 14.2	0–91.0	38.0 (30.0–50.3)	46.8 ± 17.0	0–90.0	47.0 (35.0–57.0)	**<0.001**	**<0.001**
Normal morphology, %	7.9 ± 5.8	0–30.0	6.0 (4.0–12.0)	6.1 ± 4.3	0–20.0	6.0 (2.0–8.0)	**0.006**	**0.036**
Viability, %	82.4 ± 10.0	0–98.0	85.0 (78.0–90.0)	81.1 ± 9.7	0–95.0	83.0 (78.0–88.0)	0.057	0.180

Significant *P* values are in bold.

Abbreviations: BMI, body mass index; IQR, interquartile range (25th to 75th percentiles); WC, waist circumference.

[a] In the seminal parameters analyses, one subject within each group failed to collect semen and was therefore omitted.

[b] Mann-Whitney test was used to assess differences between groups.

[c] Adjustment for multiple comparisons was calculated with the Bonferroni-Holm method.

reported; adjustment for multiple comparisons was calculated with the Bonferroni-Holm method. Spearman rank correlation coefficients were calculated to evaluate the correlations between the concentrations of each contaminant and the variables of interest.

In the gene reporter assay, to examine differences between treatment groups and positive control (T 10 nM), one-way ANOVA was performed with a Dunnett *post hoc* test, given the normal distribution of data. Due to relatively few data points per concentration and nonnormality of the data, nonparametric statistics were used to analyze the AR nuclear translocation assay. The Kruskal-Wallis test was used to compare differences between concentrations, and the Jonckheere-Terpstra test (two-tailed) was used to analyze for a linear trend between concentration and response. If one or both tests showed a significant difference ($P < 0.05$), the Mann-Whitney test with Bonferroni correction was used to compare each concentration with the control. The same procedure was applied to comparisons between different stimuli (PFOA 0.1, 1, and 10 μM and flutamide 1 μM) and T, within each T concentration.

Results

Anthropometrics and seminal measures of the two groups are reported in Table 1. In particular, subjects from the exposed group showed significantly lower mean testicular volume and shorter penile length and AGD, after adjustment for multiple comparisons. Prior to adjustments, crown-to-pubis and pubis-to-floor distances, as well as the respective ratio, differed between groups. No significant difference was observed for age and other anthropometric parameters.

Regarding seminal parameters, exposed subjects showed significantly lower sperm progressive motility and normal sperm morphology, together with higher semen pH and immotile sperm (Table 1). In addition to the reduction in semen quality, lower sperm count also was observed in exposed males, in terms of sperm concentration and total count, although not statistically significant after correction for multiple comparisons (Table 1). The overview of seminal and genital alterations is suggestive of an impairment of androgenic signaling in these subjects.

To confirm this hypothesis, we evaluated the gonadal-pituitary axis in a subset of 100 randomly chosen subjects (50 from the control group and 50 from the exposed group) who underwent also LC-MS quantification of PFOA and PFOS in serum and seminal plasma to confirm the geographic selection criteria based on exposure patterns. In the subgroup from the exposed area, increased semen pH was confirmed, together with a lower

Table 2. Sex Hormones, PFOA, and PFOS Levels in Serum and Semen From 50 Controls and 50 Exposed Subjects, With Respective Anthropometrics and Seminal Parameters

	Controls (n = 50)			Exposed (n = 50)				
Parameter	**Mean ± SD**	**Minimum–Maximum**	**Median (IQR)**	**Mean ± SD**	**Minimum–Maximum**	**Median (IQR)**	**Raw P[a]**	**Adjusted P[b]**
Serum PFOA, ng/mL	4.71 ± 2.08	1.2–8.0	4.70 (3.5–6.6)	14.99 ± 25.08	2.3–156.7	7.35 (4.7–14.9)	**<0.001**	**<0.001**
Serum PFOS, ng/mL	0.89 ± 0.7	0.6–1.8	0.82 (0.4–1.3)	1.11 ± 0.3	0.0–4.0	1.11 (0.8–1.3)	**0.012**	**0.048**
Semen PFOA, ng/mL	0.1 ± 0.01	0.0–0.1	0.1 (0.08–0.11)	0.67 ± 0.908	0.0–5.3	0.24 (0.11–0.99)	**<0.001**	**<0.001**
Semen PFOS, ng/mL	0.11 ± 0.03	0.1–0.2	0.11 (0.08–0–13)	0.12 ± 0.06	0.0–1.1	0.11 (0.01–0.14)	0.916	0.916
Testosterone, nmol/L	15.42 ± 4.06	6.8–29.4	18.98 (12.9–17.9)	19.34 ± 5.27	9.3–35.0	18.98 (16.3–21.8)	**<0.001**	**<0.001**
FSH, U/L	3.03 ± 1.26	1.2–6.6	2.89 (2.0–3.8)	3.48 ± 1.53	1.5–7.3	2.99 (2.2–7.0)	0.228	0.576
LH, U/L	4.24 ± 1.63	1.4–8.3	4.18 (2.9–6.8)	5.47 ± 1.79	2.0–8.4	5.37 (4.3–7.0)	**0.003**	**0.015**
BMI, kg/m^2	23.02 ± 2.86	18.2–33.6	22.19 (21.1–24.7)	22.90 ± 3.24	16.6–30.5	22.40 (20.4–25.5)	0.847	1.000
WC, cm	82.48 ± 8.42	64.0–114.0	80.75 (77.0–86.0)	83.13 ± 11.52	65.0–140.0	81.50 (76.5–85.4)	0.757	1.000
Arm span, cm	182.19 ± 6.61	165.0–200.0	182.75 (178.0–185.8)	180.45 ± 7.47	160.0–198.0	179.00 (174.2–187.0)	0.246	0.738
Crown-to-pubis distance, cm	81.53 ± 4.17	71.0–88.5	82.0 (79.0–85.0)	82.14 ± 4.92	74.0–96.0	82.0 (79.0–84.0)	0.592	1.000
Pubis-to-floor distance, cm	96.80 ± 5.20	87.0–106.0	97.0 (93.0–101.1)	94.59 ± 5.27	84.0–102.0	95.0 (90.3–99.0)	0.064	0.320
Crown-to-pubis/pubis-to-floor ratio	0.84 ± 0.06	0.7–1.0	0.85 (0.8–0.9)	0.87 ± 0.08	0.73–1.14	0.86 (0.8–0.9)	0.110	0.440
Testicular volume, mL	16.86 ± 3.61	9.7–26.5	16.13 (14.8–19.0)	14.67 ± 3.32	9.5–24.5	14.00 (12.6–16.0)	**<0.001**	**<0.001**
Penis length, cm	10.0 ± 1.87	6.0–13.0	10.0 (9.0–11.0)	8.75 ± 1.82	4.0–12.0	9.00 (8.0–10.0)	**<0.001**	**<0.001**
Penis circumference, cm	10.31 ± 0.90	9.0–13.0	10.10 (9.9–11.0)	9.65 ± 0.90	7.8–12.0	9.50 (9.0–10.0)	**<0.001**	**<0.001**
AGD, cm	4.5 ± 0.9	2.7–7.2	4.50 (4.0–5.2)	4.2 ± 0.8	2.5–5.7	4.00 (3.5–5.0)	**0.019**	0.114
Semen volume, mL	3.09 ± 1.91	0.5–9.0	2.55 (1.5–3.9)	2.76 ± 1.37	0.3–6.0	3.00 (1.6–3.4)	0.373	1.000
pH	7.55 ± 0.21	7.0–7.9	7.60 (7.5–7.7)	7.62 ± 0.23	7.0–8.0	7.70 (7.6–7.7)	**0.005**	**0.042**
Sperm concentration, 10^6/mL	92.40 ± 133.87	6.0–800.0	49.50 (27.4–94.3)	89.76 ± 59.35	6.0–264.0	54.50 (34.4–96.5)	0.771	1.000
Total sperm count, 10^6	241.78 ± 347.40	6.1–2240	146.25 (70.0–270.3)	226.93 ± 264.38	15.8–680	171.55 (77.2–301.7)	0.596	1.000
Progressive motility, %	54.73 ± 13.39	30.0–85.0	54.50 (45.0–64.8)	55.31 ± 16.41	15.0–88.0	57.0 (43.0–68.0)	0.992	1.000
Nonprogressive motility, %	6.88 ± 6.38	0.0–27.0	4.50 (3.0–9.8)	5.06 ± 3.35	1.0–21.0	4.0 (3.0–6.0)	0.106	0.636
Immotile sperm, %	39.08 ± 12.34	15.0–68.0	38.0 (30.2–46.5)	39.63 ± 16.49	7.0–79.0	40.50 (25.5–53.5)	0.624	1.000
Normal morphology, %	8.72 ± 5.51	2.0–20.0	7.0 (4.0–12.0)	4.55 ± 2.31	2.0–10.0	4.0 (2.0–6.0)	**<0.001**	**<0.001**
Viability, %	82.44 ± 7.81	53.0–92.0	82.50 (80.0–89.5)	79.69 ± 7.67	60.0–91.0	82.0 (75.0–85.0)	**0.048**	0.336

Significant *P* values are in bold.

Abbreviations: BMI, body mass index; IQR, interquartile range (25th to 75th percentiles); WC, waist circumference.

[a] Mann-Whitney test was used to assess differences between groups.

[b] Adjustment for multiple comparisons was calculated with the Bonferroni-Holm method.

percentage of sperm with normal morphology, reduced penile length and circumference, and smaller testicular volume, but not AGD, although only after adjustment for multiple comparisons (Table 2). No significant difference has emerged in terms of other seminal or anthropometric parameters. PFOA was detected in serum from 98% subjects and in 96% of the respective seminal plasma, whereas PFOS was detected in 90% of sera and 86% of seminal plasma. PFC quantification has confirmed higher serum levels of both PFOA and PFOS in exposed subjects (Table 2), with the former being the prominent species in blood, with a mean of 14.99 ng/mL in the exposed group and 4.71 ng/mL in control subjects. In addition, the concentration of PFOA, but not PFOS, was higher in the seminal plasma from exposed subjects, although lower than serum levels (Table 2). Hormonal analyses showed higher levels of total T and LH in the exposed group, compared with control counterparts (Table 2). In the correlation analyses, serum and seminal plasma levels of PFOS and PFOA were highly correlated with each other (Spearman $\rho = 0.216$, $P = 0.034$ and $\rho = 0.294$, $P = 0.003$, respectively), as were PFOA plasma and semen concentrations ($\rho = 0.449$, $P < 0.001$), but not PFOS plasma and semen levels ($\rho = 0.163$, $P = 0.111$). Serum PFOA levels were positively correlated with total T ($\rho = 0.305$, $P = 0.002$; Fig. 1) and LH ($\rho = 0.224$, $P = 0.046$), as were seminal PFOA ($\rho = 0.346$, $P < 0.001$ and $\rho = 0.259$, $P = 0.021$), and with the proportion of sperm with normal morphology ($\rho = -0.303$, $P = 0.002$ and $\rho = -0.225$, $P = 0.025$, respectively). Again, seminal PFOA showed a positive correlation with pH ($\rho = 0.203$, $P = 0.042$). Both serum and semen PFOA, but not PFOS, were associated with reduced testicular volume ($\rho = 0.211$, $P = 0.037$ and $\rho = -0.277$, $P = 0.006$, respectively).

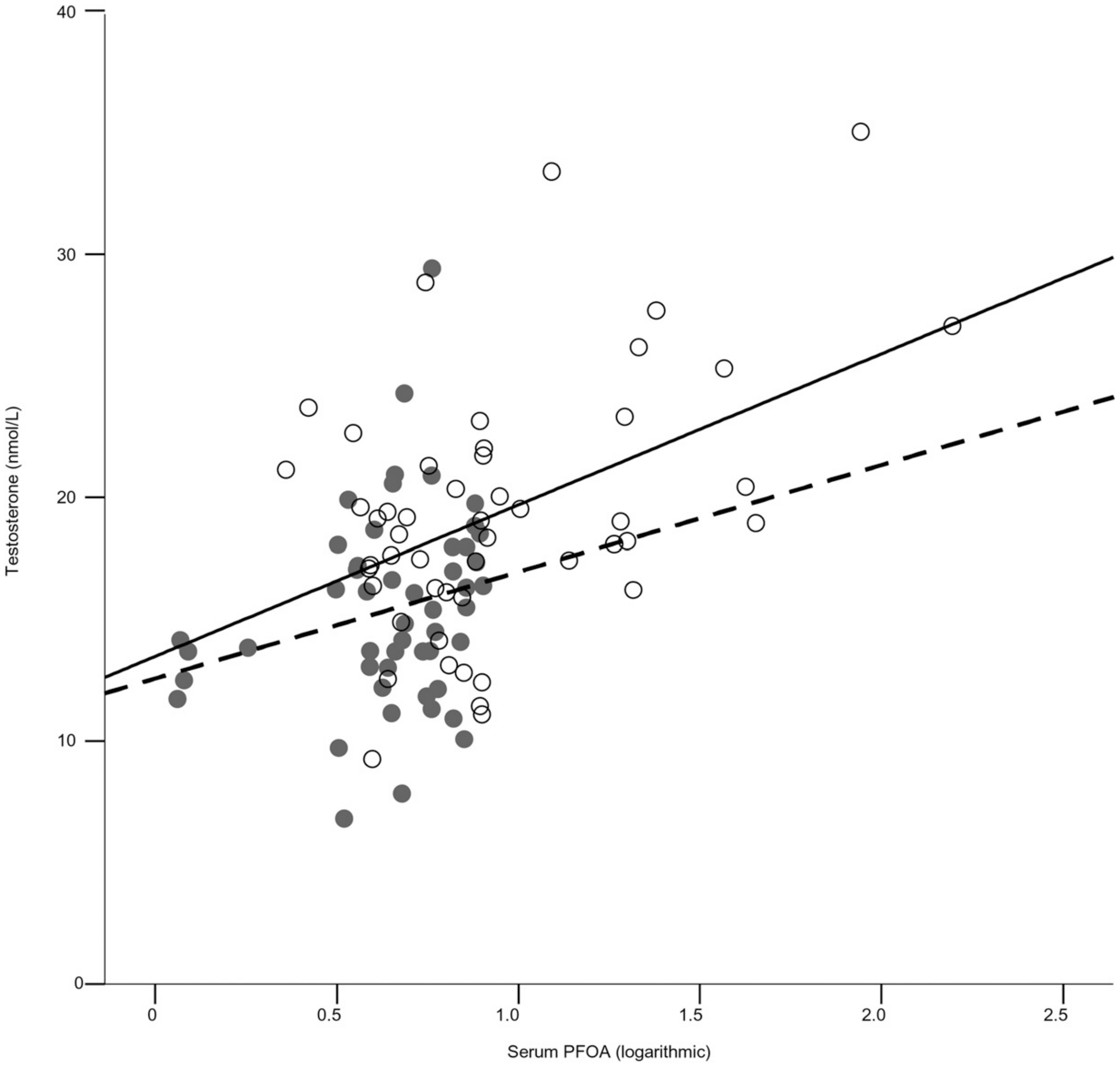

Figure 1. Total testosterone levels are positively correlated with serum PFOA. Correlation between serum log-transformed PFOA levels and total T in the exposed group (full circles, straight line, n = 50) and in control group (empty circles, dotted line, n = 50).

Within this framework of clinical signs suggestive of an endocrine disruption of androgen action by PFCs, we aimed to experimentally test the hypothesis of an interference of these chemicals on the AR, the mediator of androgen signaling. To this end, an AR gene reporter assay on HeLa cells transiently cotransfected with an MMTV-LUC reporter vector and an AR expression plasmid pSV-AR0. PFOS and PFOA at a concentration of 1 μM acted as mild agonists of AR (10.5% and 11.6%; Fig. 2). Upon coincubation with T 10 nM, both PFOS and PFOA elicited a significant ($P < 0.001$) antagonistic effect on T-induced activation of AR at concentrations comparable with those reported in highly exposed populations. These compounds antagonized the T-induced response (set to 100%) down to 73.5% and 64.2%, respectively (Fig. 2), with PFOA being the most potent AR inhibitor. The relative potencies of the tested compounds were approximately twice lower than the inhibitor control flutamide (Fig. 2).

Given the highest occurrence of PFOA in the serum of exposed Italian populations and its higher potency compared with PFOS in the gene reporter assay, we focused on PFOA to elucidate the antiandrogenic mechanism of PFCs. SPR measurements were performed to monitor the real-time interaction between T and PFOA with the AR. In this experiment, the AF2 binding domain of AR was immobilized on a CM5 sensor chip, and solutions of T (0 to 1 mM) and PFOA (0 to 4 μM) were injected separately at different concentrations. Despite the low molecular weight of T (288 Da), SPR resolved the interaction and provided a dissociation constant K_d = 174 ± 32 μM (Fig. 3A and 3B). In contrast, no

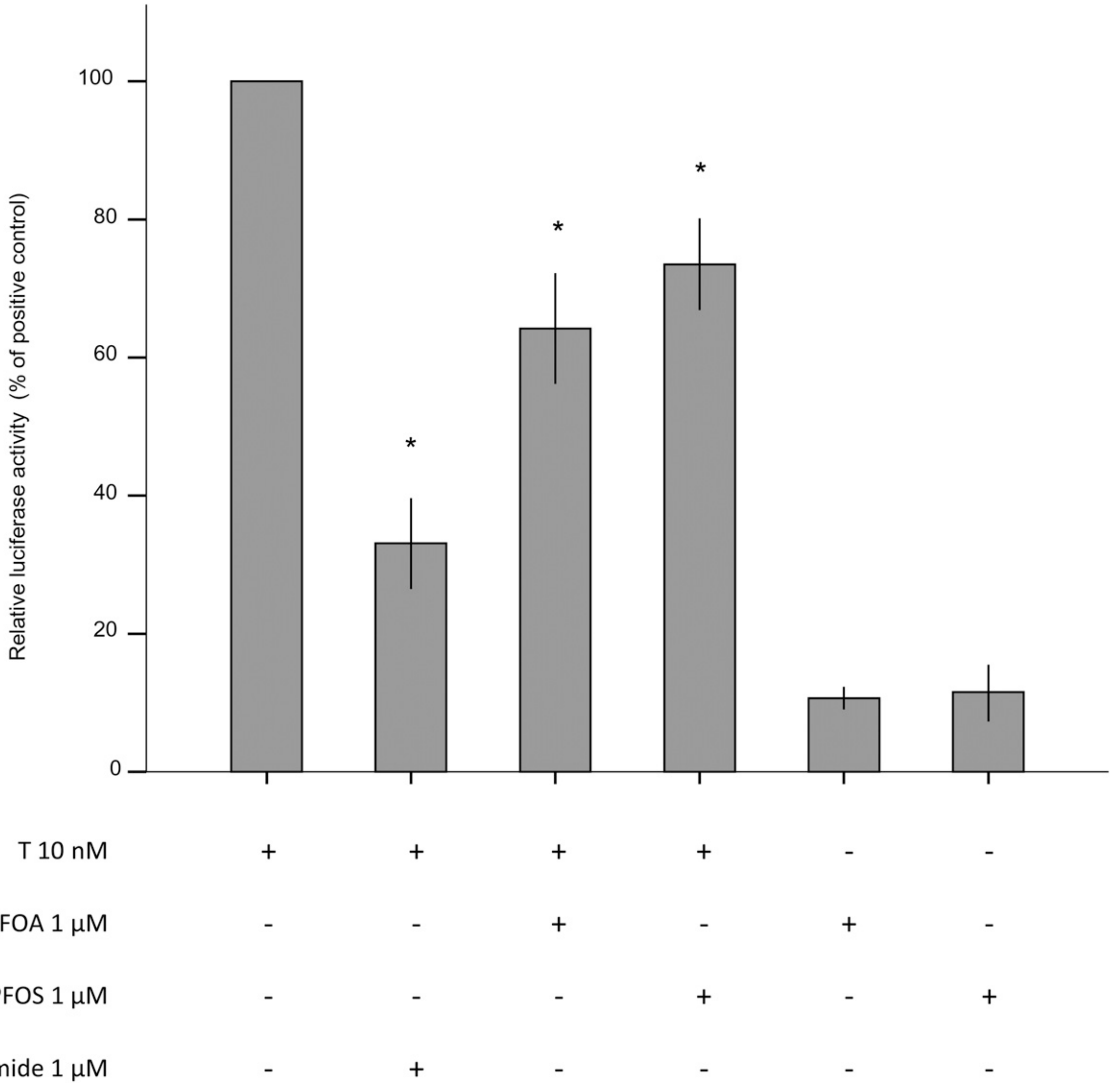

Figure 2. PFOA and PFOS inhibit AR transactivation in HeLa transfected cells. AR gene reporter assay on HeLa cells transfected with Luc-AR and stimulated with T 10 nM and PFOA 1 μM or PFOS 1 μM, alone or in combination with T. Flutamide (Flut 1 μM) served as internal negative control of inhibitory activity on AR. AR activity is reported as relative (%) to positive control (T 10 nM, set to 100%). Data are reported as mean ± SD of three independent experiments. *$P < 0.001$ calculated with one-way ANOVA with a Dunnett *post hoc* test with T 10 nM as reference category.

interaction between PFOA (up to 4 μM) and AR was detected under the same experimental conditions (Fig. 3C and 3D). Higher concentrations of PFOA were not tested because of the limited solubility in the running buffer. Next, we performed a competition experiment to assess whether the presence of PFOA would reduce the binding of T to AR. We incubated a solution of T with different concentrations of PFOA, and the resulting mixture was flowed over the same AR-coated sensor chip. At the highest concentration tested, we observed a small but significant (35%) decrease of T binding, suggesting that the presence of PFOA reduces the binding of T to its receptor (Fig. 3E).

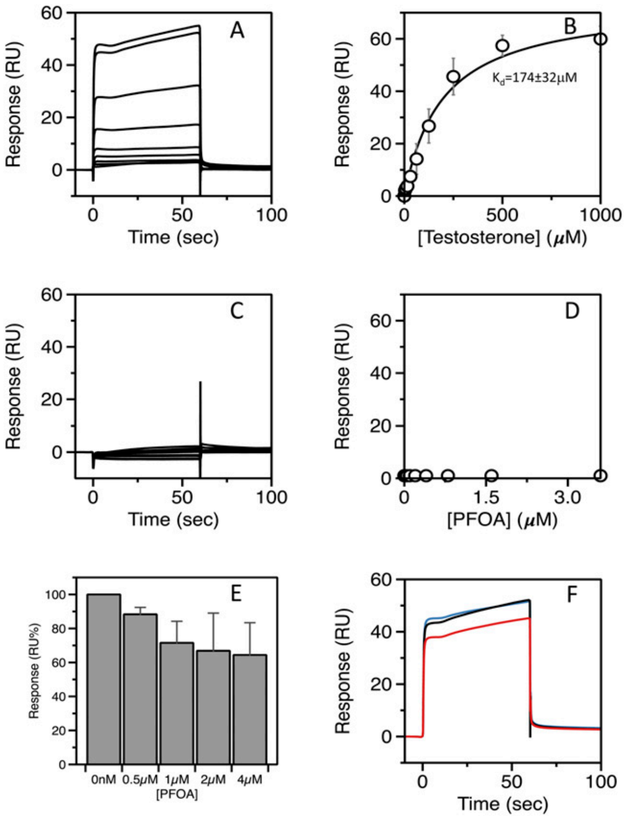

Figure 3. Binding of testosterone to immobilized androgen receptor is reduced by PFOA. Solutions of (A) testosterone (288.42 Da) and (C) PFOA (414.07 Da) were injected at a flow rate of 30 μL/min at 25°C, using 10 mM Hepes (pH 7.4), 0.15 M NaCl containing 3% MeOH (v/v) as running buffer. Each SPR trace was subtracted for unspecific binding ($<2\%$ of RU$_{max}$). (B, D) The response units (RUs) at the steady state were plotted as a function of [analyte] and fitted to the Langmuir equation to yield the dissociation constant K$_d$. No interaction between PFOA (up to 4 mM) and AR was detected under the same experimental conditions. (E) Next, we performed a competition experiment to assess whether the presence of PFOA would reduce the binding of testosterone to AR. A 250-mM solution of T was incubated with different concentrations of PFOA (0 to 4 μM) for at least 10 minutes before injection over the same AR-coated sensor chip. We observed a ~35% reduction of T binding to AR at 4 μM PFOA. Results are shown as the maximal association RUs (expressed as the percentage relative to the response measured without PFOA) achieved at increasing concentrations of PFOA. (F) Raw data showing the inhibitory effect of PFOA at 1 μM (red) and the reproducibility of T binding before and after the competition experiment (black and blue).

In a final set of experiments, we aimed to test *in vitro* the putative inhibitory effect of PFOA on AR. To this end, a nuclear translocation assay was performed on murine Leydig MA-10 cells, cultured at different concentrations of T (1 to 100 nM) and PFOA (0.1 to 1 μM), alone or in combination. Flutamide 1 μM served as a negative control. In the positive control, T elicited a significant (all $P < 0.001$ vs unstimulated cells) and consistent AR nuclear internalization, with ~90% of positive signal within the nucleus, even at the lowest concentration (Figs. 4 and 5). By addition of androgen inhibitor flutamide, AR nuclear signal decreased down to 17.9%, 20.9%, and 36.4% at T concentrations of 1, 10, and 100 nM, respectively. A very low signal was detected in cells incubated with PFOA 0.1, 1, or 10 μM (2.4%, 3.4%, and 7.1%, respectively; Fig. 4), which, however, was comparable with that of the negative unstimulated control (4.1%). When Leydig cells were coincubated with both PFOA and T, a dose-dependent inhibition of AR nuclear translocation was observed for increasing concentrations of PFOA, which was inversely correlated to T concentration (Fig. 4 and 5): at the highest T concentration (100 nM), PFOA did not affect AR internalization at any concentration, but at physiologically relevant T levels (10 nM), AR nuclear signal significantly decreased at the highest PFOA concentration (10 μM). On the other hand, at lower levels of T (1 nM), PFOA induced a significant reduction of AR internalization at any tested concentration (Fig. 5). A Jonckheere-Terpstra test for ordered alternatives showed that there was a statistically significant trend of higher AR nuclear translocation scores with increasing concentration of T, alone ($z = 2.416$, $P = 0.016$) or in combination with flutamide ($z = 3.695$, $P < 0.001$) and PFOA, at any tested concentration (all $P < 0.001$).

Discussion

This study documents that PFCs have a substantial impact on human male health as they directly interfere with hormonal pathways, potentially leading to male infertility. We found that increased levels of PFCs in plasma and seminal fluid positively correlate with circulating T and with a reduction of semen quality, testicular volume, penile length, and AGD. Experimental evidence supports our observational results and points toward an antagonistic action of PFOA on the binding of T to its natural AR.

The investigation covered an area of around 150 km^2 in the provinces of Vicenza and Padua and, to some extent, Verona, with 350,000 to 400,000 people potentially

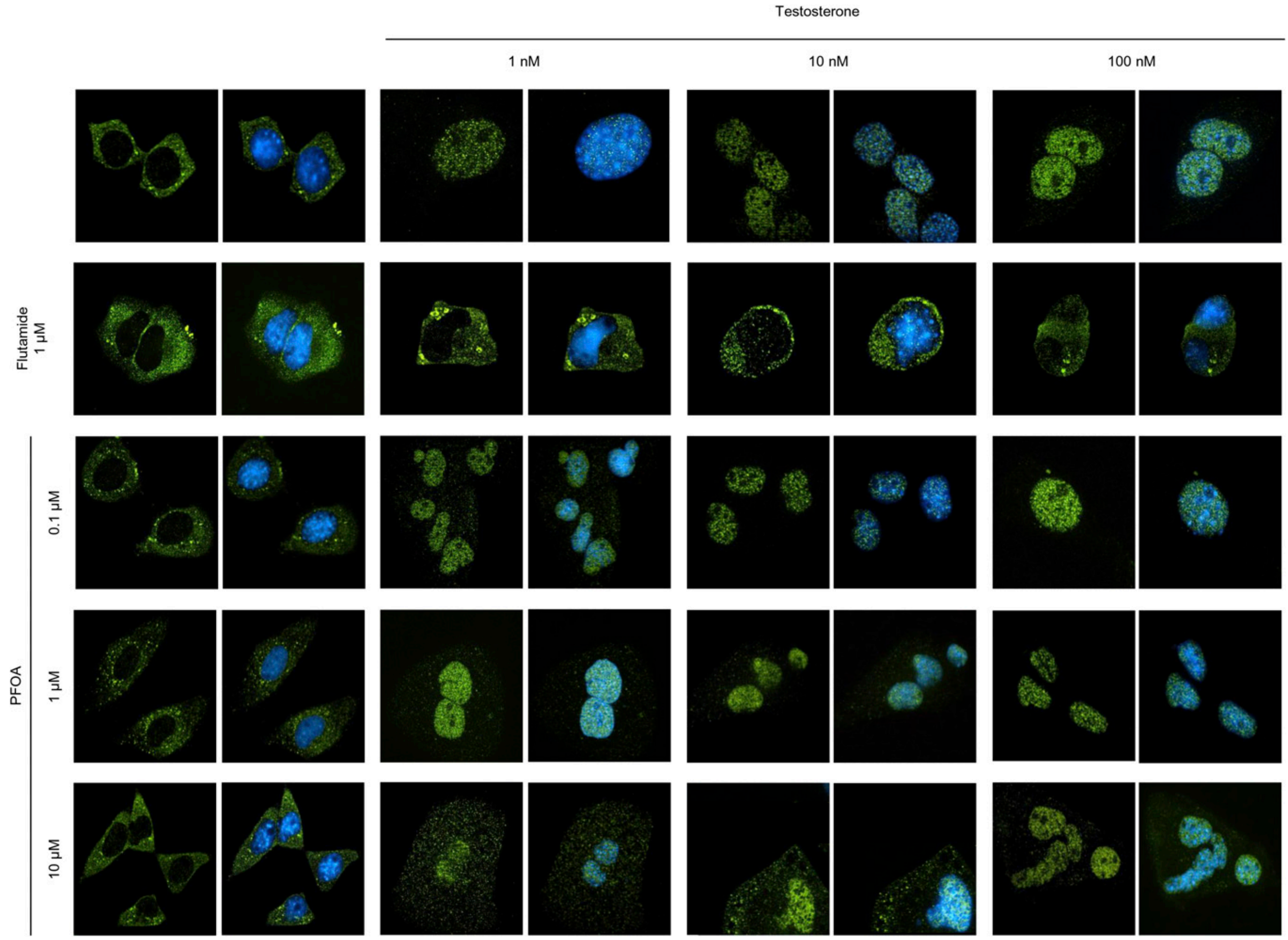

Figure 4. Expression of AR in murine Leydig MA-10 cells under different conditions. Immunofluorescence of AR (FITC, green) nuclear translocation in Leydig Ma-10 cells stimulated with T and PFOA at different concentrations as reported in the figure. Flutamide (1 μM) served as internal control. Nuclei are stained with 4′,6-diamidino-2-phenylindole (blue). Cells were visualized by scanning confocal laser microscopy (×60 magnification).

exposed (35, 36). These areas are heavily polluted with concentrations of PFCs up to 6872 ng/L for all PFCs and up to 3733 ng/L for PFOA alone in surface waters and up to 3138 ng/L for all PFCs and up to 1886 ng/L for PFOA in drinking water, which is >1000-fold higher than control values (0.5 to 8 ng/L) (37). Compared with median concentrations of PFOA and PFOS in blood serum of the general population in Italy, we found levels of PFOA more than five times higher in plasma and semen compared with control. Although slightly inferior to the PFC levels calculated on 13,856 subjects aged 14 to 40 years, during a surveillance program promoted by Veneto from the most polluted "red zone" (38), our results are consistent with previous findings (35), and our sensitive LC-MS method is able to differentiate between exposed population and controls.

Interestingly, most of the exposed male population showed a reduction in testicular volume, penile length, and AGD but not anthropometrics in males aged 18 to 19 years. These findings could be explained by considering that AGD and anthropometric measures are differentially determined during fetal and prepubertal development, respectively (24). Accordingly, genital development is concomitant with AGD determination (39). Therefore, we could speculate a hypothetic involvement of PFC to *in utero* rather than late ED exposure. Prenatal exposure to androgens during the "masculinization programming window," a critical window during testicular development, is positively associated with AGD in mammals (39). On these bases, AGD has been suggested as a putative marker of prenatal exposure to chemicals with a known antiandrogenic effect, or ED in general. For example, exposure to phthalates (40), dioxins (41), and bisphenol A (42) has been associated with a reduction in AGD. As the first report on water contamination of PFCs goes back to 1977 (43), the magnitude of the problem is alarming as it affects an entire generation of young individuals, from 1978 onward.

PFC toxicity also concerns adult life independently from *in utero* exposure. This implies that healthy

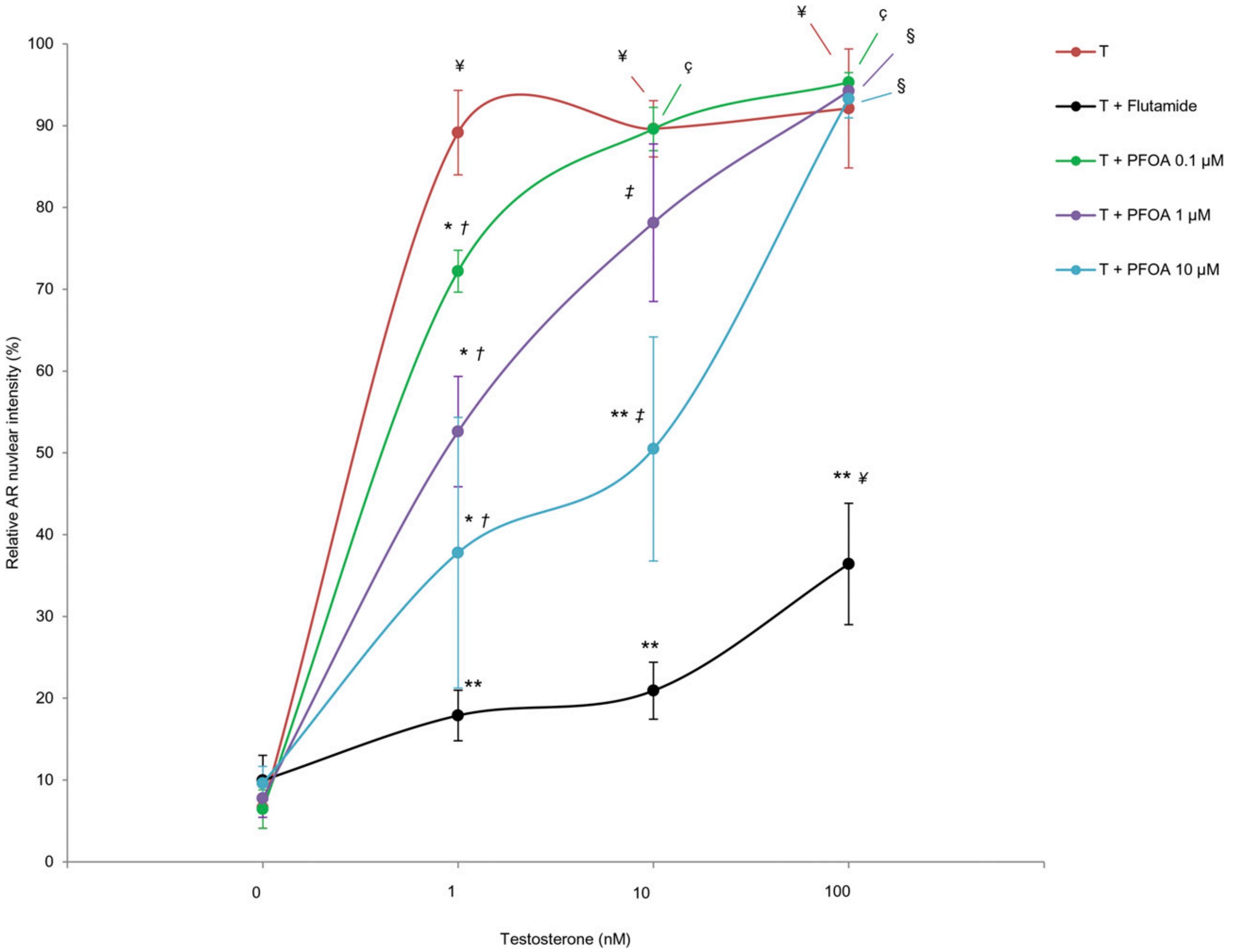

Figure 5. AR nuclear translocation induced by T is reduced by PFOA in murine MA-10 Leydig cells. Relative quantification (%) of nuclear fluorescence intensity with respect to total fluoresce intensity in MA-10 Leydig cells stimulated with T and PFOA at different concentrations. Results are the mean ± SE. The Kruskal-Wallis test was used to compare differences between concentrations. The Mann-Whitney test with Bonferroni correction was used to compare each concentration with the control. The same procedure was applied to comparisons between different stimuli (PFOA 0.1, 1, and 10 μM and flutamide 1 μM) and T, within each T concentration. $^{*}P < 0.05$ vs T; $^{**}P < 0.001$ vs T; $^{\dagger}P < 0.05$ vs T 0, 10, and 100 nM; $^{\ddagger}P < 0.05$ vs T 0, 1, and 100 nM; $^{\S}P < 0.05$ vs T 0, 1, and 10 nM; $^{¥}P < 0.05$ vs T 0 nM; $^{ç}P < 0.05$ vs 0 and 1 nM.

individuals living in territories contaminated with PFCs could present signs of toxicity. *In vitro* and animal studies on PFC toxicity have shown a detrimental effect of PFCs on testicular function due to the alteration of steroidogenic machinery and subsequent defect of spermatogenesis (44–48). Two cross-sectional studies reported negative associations of PFOS, or high PFOA and PFOS combined, with the proportion of morphologically normal spermatozoa in adult men (49, 50). This is in agreement with our findings, in which we observed a significant reduction in progressive sperm motility in exposed subjects. The exact mechanism, however, is not clear and possibly involves an impairment of mitochondrial activity, as observed in the endocrine disruptor bisphenol A (51).

Another important finding is the association between PFOA and seminal plasma pH, indicative of an interference of PFCs at a prostatic level. The presence of PFCs in seminal plasma reported by previous groups (52) and by us suggests either a prostatic or testicular origin of PFCs that could explain a weak association of plasma PFOS concentration with incident prostate cancer (53). This aspect, however, requires further investigations.

Overall, the inefficient recognition between T and its receptor in the presence of PFCs could explain the clinical symptoms in the exposed individuals. It would also explain why higher levels of T are found in exposed subjects, which is a compensatory mechanism, as supported by increased LH. Interestingly, in the only study that evaluated young males from an exposed pregnancy cohort, prenatal exposure to PFOA was associated later in adult life with lower sperm concentration and total sperm count (16). The same study also reported an alteration of the hypothalamic-pituitary axis, with higher levels of LH and FSH but not T.

Several experiments provide direct evidence that PFOA inhibits the binding of T to AR. First, PFOS and PFOA elicit a significant antagonistic effect (~25%) on testosterone-induced activation of AR in HeLa cells. This result is in agreement with the study by Kjeldsen and Bonefeld-Jørgensen (18) but in contrast with Behr *et al.* (54) and Du *et al.* (55), who have used different cell lines, reporter plasmids, and cotreatment conditions. Second, PFOA diminishes the binding of T to the purified receptor. Third, PFOA significantly reduces the translocation of AR to the nucleus in murine Leydig cells. Remarkably, coincubation of physiological concentrations of T in adults (10 nM) and PFOA led to a ~20% reduction of AR nuclear signal, at concentrations reported in regions with point source drinking water contamination (1 μM) (35, 36, 56) and in occupationally exposed fluorochemical workers (10 μM) (57).

Despite the convincing biological effect, the mechanism of inhibition remains elusive and requires more biochemical investigations to be unveiled. Moreover, quantification of circulating androgens with more precise methods could unveil further associations with sex steroids, and given the cross-sectional design of the study, further confounding factors could be included, such as socioeconomic status. Because of the partial antagonist effect in our assays, PFOA may act as an allosteric or noncompetitive inhibitor, thereby blocking dimerization of the receptor and its translocation to the nucleus. This would explain why SPR experiments failed to monitor the interaction between PFOA and covalently immobilized monomeric AR. Alternatively, PFOA could interact with T, thereby diminishing the concentration of the bioactive hormone in the circulation. Furthermore, it remains to be established how PFCs penetrate cells and barriers and what are the mechanisms of clearance.

In conclusion, we present both clinical and experimental evidence supporting the endocrine-disrupting activity of PFCs on androgenic function, which is mediated by the AR. The interference of PFCs on the binding and activation of T on the AR could explain the resulting alterations of seminal parameters and the reduction in testicular volume and penile length, together with shorter AGD, observed in young males from an exposure area. At the hormonal level, the reduced activation of T results in increased serum T levels, possibly due to the positive feedback on the hypothalamic-pituitary axis, as reflected by increased LH. Importantly, the antagonistic activity on T by PFCs could also extend to other steroids, such as DHT, progesterone, or estradiol, thereby affecting early and late development of the male genital tract to different extents.

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