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Fluorinated alkyl substances and technical mixtures used in food paper-packaging exhibit endocrine-related activity in vitro

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SUMMARY

Migration of chemicals from packaging materials to foods may lead to human exposure. Polyfluoroalkyl substances (PFAS) can be used in technical mixtures (TMs) for use in food packaging of paper and board, and PFAS have been detected in human serum and umbilical cord blood. The specific structures of the PFAS in TMs are often unknown, but polyfluorinated alkyl phosphate esters (PAPs) have been characterized in TMs, food packaging, and in food. PAPs can be metabolized into fluorotelomer alcohols (FTOHs) and perfluoroalkyl carboxylic acids (PFCAs). Some PFAS have endocrine activities, highlighting the need to investigate these effects. Herein, we studied the endocrine activity of less characterized PFAS, including short-chain PFCAs and FTOHs, PAPs, and TMs of unknown chemical composition. Long-chain PFCAs were also included. We applied seven assays covering effects on estrogen, glucocorticoid, androgen, and peroxisome proliferator-activated receptor (PPAR) activity, as well as steroidogenesis in vitro and ex vivo. In general, PAPs, FTOHs, TMs, and long-chain PFCAs showed estrogenic activity through receptor activation and/or increasing 17β-estradiol levels. Furthermore, short- and long-chain PFCAs activated PPARα and PPARγ. Collectively, this means that (i) PAPs, FTOHs, and PFCAs exhibit endocrine activity through distinct and sometimes different mechanisms, (ii) two out of three tested TMs exhibited estrogenic activity, and (iii) short-chain FTOHs showed estrogenic activity and short-chain PFCAs generally activate both PPARα and PPARγ with similar potency and efficacy as long-chain PFCAs. In conclusion, several new and divergent toxicological targets were identified for different groups of PFAS.

INTRODUCTION

The road from food production to consumption can be long and complex. It involves multiple steps where chemical contamination of foodstuff can occur. Food packaging materials are potential sources of such contamination (Borchers *et al.*, 2010). For example, food packaging of paper and board can contain chemicals originating from printing inks, adhesives and coatings. If these chemicals migrate to the food, there is a risk for human exposure that may cause adverse health effects, particularly because they inevitably will be present in combination with other chemicals from various sources.

Among chemicals used in food packaging materials of paper and board are polyfluoroalkyl substances (PFAS). These are added to technical mixtures (TMs) used for coatings or sizing agents to impart water and oil resistance to the material (Kissa, 2001). Polyfluorinated alkyl phosphate esters (PAPs) constitute one group of PFAS which has been found in TMs, food packaging, and food (Begley *et al.*, 2005, 2008; Tittlemier *et al.*, 2006; Stahl *et al.*, 2011; Trier *et al.*, 2011; Gebbink *et al.*, 2013), but TMs containing other PFAS are also available on the market. The specific structures of the PFAS in many TMs are unknown, which pose challenges with respect to assessing toxicity and exposure, and ultimately their risk to humans. Paper and board food packaging is not covered by a specific regulation in Europe, however a general regulation exists stating that the compound must not be transferred to food in amounts that can adversely affect human health (The European Commission 2004). To our knowledge, some TMs and PFAS for use in paper and board are

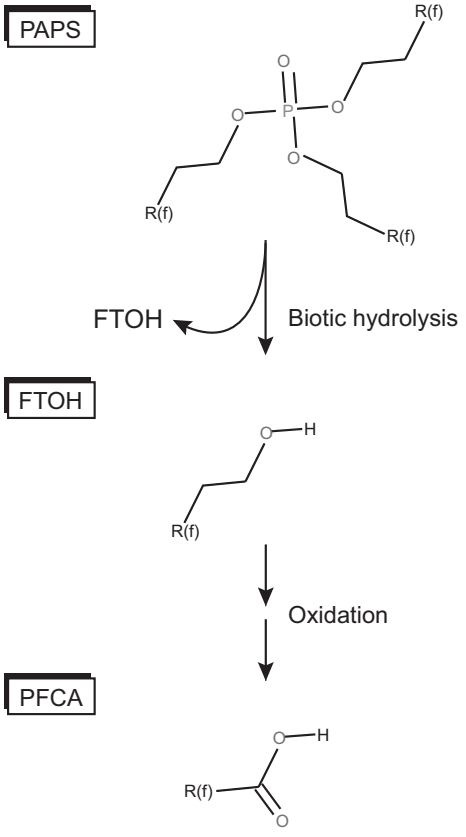
presently covered by regulations for instance in the US and Germany (BfR 2015, US FDA, 2015).

PFAS synthesized from fluorotelomer alcohols (FTOHs) can degrade to perfluoroalkyl carboxylic acids (PFCAs) during production, use and disposal (Scheringer *et al.*, 2014). Consequently, FTOHs and PFCAs may be present in TMs as impurities (Begley *et al.*, 2005; Prevedouros *et al.*, 2006). PAPs can also be metabolized into PFCAs, likely through the intermediate metabolites FTOHs (Fig. 1) (D'eon & Mabury, 2007, 2011). Thus, PFCAs and FTOHs represent potential direct and indirect sources of exposure. Indeed, both PAPs and the final metabolites have been detected in human blood (Houde *et al.*, 2006; Calafat *et al.*, 2007; D'eon *et al.*, 2009; Olsen *et al.*, 2012) and breast milk (So *et al.*, 2006; Kubwabo *et al.*, 2013), with PFCAs also detected in umbilical cord blood (Monroy *et al.*, 2008; Kim *et al.*, 2011). This means that humans could be exposed to PFAS during fetal, neonatal, as well as adult life. This is of general concern considering the long half-life of some PFAS in human blood (Olsen *et al.*, 2007), and of particular concern in relation to fetal exposure because endocrine disrupting chemicals may contribute to disrupted development leading to compromised health at birth or later in life (Skakkebaek *et al.*, 2001). These concerns have recently led Denmark to introduce a guidance limit-value for the sum of PFAS in food packaging materials (The Danish Veterinary and Food Administration, Ministry of Environment and Food, 2015).

Some PFAS are reported to have endocrine disruptive potential, interfering with both the thyroid and steroid hormone systems (Lau, 2012). Effects on steroid hormones include increased 17β-estradiol levels in blood following exposure to some PFCAs and FTOHs (Cook *et al.*, 1992; Biegel *et al.*, 1995, 2001; Feng *et al.*, 2009; Liu *et al.*, 2009, 2010) and decreased testosterone levels following in vivo exposure to some PFCAs (Bookstaff *et al.*, 1990; Cook *et al.*, 1992; Shi *et al.*, 2010). Exposure to PAPs can result in increased estrogen and decreased androgen levels in vitro (Rosenmai *et al.*, 2013). Additionally, many PFCAs interfere with the activity of peroxisome proliferator-activated receptor α (PPARα) (Wolf *et al.*, 2008, 2012; Buhrke *et al.*, 2013), a mechanism associated with tumour development in the liver, pancreas and testicles (Lau, 2012). PFCAs also activate the PPARγ (Buhrke *et al.*, 2013; Zhang *et al.*, 2014), a receptor which plays a role in preadipocyte differentiation into adipocytes (Ferré, 2004). Thus, PFAS may be involved in the development of obesity through affecting this receptor.

Notably, the above-mentioned studies typically analysed long-chain PFAS, whereas short-chain PFAS and fluorinated constituents of some TMs remain poorly characterised with respect to their ability to interfere with hormone systems. This is of particular concern because the industry has moved from long-chain to short-chain chemistry (Scheringer *et al.*, 2014), meaning that humans will be increasingly exposed to short-chain PFAS. PFHxA and PFBA have already been detected in several types of foods (Pérez *et al.*, 2014) and in liver, brain, kidney and/or lung tissue of twenty post-mortem individuals (Pérez *et al.*, 2013). Therefore, we sought to gain new insight into the endocrine activity of short-chain PFAS and TMs by assessing their in vitro activity across a range of established endocrine-related assays. We analysed short-chain PFCAs of chain lengths 4–7, three FTOHs, four PAPs, as well as three TMs of unknown chemical composition. Additionally, we included PFCAs of longer chain lengths from 8 to 12. The combination of substances enabled comparison between effects based on length of fluorinated chain, degree of fluorination, presence of hydrocarbon groups in the substance, and the role of the functional head group.

Figure 1 Metabolic pathway of PAPs into the intermediate metabolites FTOHs and the final metabolite PFCAs (D'eon & Mabury, 2007; D'eon & Mabury, 2011). RF = $F(CF_2)_n$. In this study: diPAPs (n = 8 or 10), monoPAP and triPAP (n = 8), FTOHs (n = 4, 6, or 8), PFCAs (n = 3–11).



MATERIALS AND METHODS

Test substances and technical mixtures

All substances used in the study are listed in Table 1 together with their respective elemental composition, CAS numbers and reported purity. In addition, three commercially available fluorine-based TMs for use in food contact materials were tested and are denoted TM1, TM2, and TM3, corresponding to Solvera PT5045 (Solvay Solexis, Bollate, Italy) [fluoropolyether ammonium phosphate salt, 16–20% dry matter (measured 23.2%)], Capstone P-620HS (DuPont de Nemours, Leiden, the Netherlands) [fluorinated acrylic cationic copolymer, total fluorine content 25 ± 1% (measured 27.3%)], and Cartafluor CFI (Clariant presently owned by Archroma, Vienna, Austria) (fluorinated acrylic cationic copolymer, measured dry matter 40.8%), respectively. Although the overall chemical structure was given, the specific structures, the distribution of the homologues series and the concentrations in the commercially available TMs were unknown.

Stock solutions were prepared by dissolving PFdoDA and TM3 in ethanol, TM1 and TM2 in H_2O, and all other substances in dimethyl sulfoxide (DMSO). PFdoDA was dissolved in ethanol as it was not dissolvable in DMSO at the desired concentration,

Table 1 Names, abbreviations, CAS numbers and purity of test compounds

Abbreviation	Name	Elemental composition	CAS	Purity (%)
4 : 2 FTOH	4 : 2 fluorotelomer alcohol	$F(CF_2)_4(CH_2)_2OH$	2043-47-2	97
6 : 2 FTOH	6 : 2 fluorotelomer alcohol	$F(CF_2)_6(CH_2)_2OH$	647-42-7	97
8 : 2 FTOH	8 : 2 fluorotelomer alcohol	$F(CF_2)_8(CH_2)_2OH$	678-39-7	97
PFBA	Perfluorobutanoic acid	$F(CF_2)_3COOH$	375-22-4	98
PFPeA	Perfluoropentanoic acid	$F(CF_2)_4COOH$	375-85-9	97
PFHxA	Perfluorohexanoic acid	$F(CF_2)_5COOH$	307-24-4	97
PFHpA	Perfluoroheptanoic acid	$F(CF_2)_6COOH$	375-85-9	99
PFOA	Perfluorooctanoic acid	$F(CF_2)_7COOH$	335-67-1	96
			3825-26-1	98
PFNA	Perfluorononanoic acid	$F(CF_2)_8COOH$	375-95-1	97
PFDA	Perfluorodecanoic acid	$F(CF_2)_9COOH$	335-76-2	98
PFunDA	Perfluoroundecanoic acid	$F(CF_2)_{10}COOH$	2058-94-8	95
PFdoDA	Perfluorododecanoic acid	$F(CF_2)_{11}COOH$	307-55-1	97
8 : 2 monoPAP	8 : 2 polyfluoroalkyl phosphate ester	$F(CF_2)_8(CH_2)_2OP(O)OH_2$	57678-03-2	99.5
8 : 2 diPAP	8 : 2/8 : 2 polyfluoroalkyl phosphate ester	$[F(CF_2)_8(CH_2)_2O]_2P(O)OH$	678-41-1	98
10 : 2 diPAP	10 : 2/10 : 2 polyfluoroalkyl phosphate ester	$[F(CF_2)_{10}(CH_2)_2O]_2P(O)OH$	1895-26-7	94.6
8 : 2 triPAP	8 : 2/8 : 2/8 : 2 polyfluoroalkyl phosphate ester	$[F(CF_2)_8(CH_2)_2O]_3P(O)$	NA	99.5

NA, Not available.

whereas the solvents for the TMs were based on recommended solvents according to technical datasheets supplied by the manufacturers. Notably, the TMs are of unknown composition, and it is therefore unclear whether all components are dissolved. However the stated fluorinated constituents, fluoropolyether ammonium phosphate salts and fluorinated acrylic cationic copolymers, as well as some impurities including FTOHs and PFCAs are soluble in water and/or ethanol and should thus be in solution. Nevertheless, results for the TMs should be interpreted with caution. Concentrations of stock solutions were 20 mM for PAPs and 40 mM for all remaining substances. Stated intervals of substances, for example, PFBA-PFHpA, denotes an interval of chain-lengths with increasing number of repeats of CF_2 units, in this case, the substances PFBA, PFPeA, PFHxA and PFHpA.

Steroidogenesis assay

The steroidogenesis assay was performed using the NCI-H295R human adrenal corticocarcinoma cell-line (ATCC) as described previously (Rosenmai *et al.*, 2013). Briefly, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Life Technologies, Bleiswijk, the Netherlands) with 1% Insulin–Transferrin–Sodium selenite plus premix (VWR) and 2.5% Nu-Serum (BD Bioscience, Brøndby, Denmark). Cells were seeded in 24-well plates at a density of 3×10^5 cells/well and left to grow for 24 h before exposure. Cells were exposed to test compounds for 48 h with a successive sampling of medium for hormone analysis. The MTT assay was performed on cells after medium removal as described previously (Rosenmai *et al.*, 2013) to assess cell viability. Forskolin (1 and 10 μM) (Sigma-Aldrich, Copenhagen, Denmark) and prochloraz (0.3 and 3 μM) (Ehrenstorfer, VWR & Bie & Berntsen, Denmark) were included in all experiments to monitor assay performance. Extraction and quantification of progesterone, testosterone and 17β-estradiol (Delfia™ time-resolved fluoroimmunoassays; PerkinElmer, Skovlunde, Denmark), were performed as previously described (Vinggaard *et al.*, 2002; Rosenmai *et al.*, 2013). For PFDA-PFdoDA, a fluorescamine cell viability assay was conducted, as an unexplained increased response was observed in some of the MTT assays. The assay was performed by washing cells with PBS, adding 150 μL lysis buffer (25 mM trisphosphate pH 7.8, 15% glycerol, 1% Triton-X, 1 mM 1,4-Dithiothreitol (DTT)), followed by addition of 600 μL of a 1.8 mM fluorescamine solution to each well. Successively, plates were incubated for 20 min, shaken and then fluorescence measured with excitation wavelength 390 nm and emission 460 nm. Substances causing increased 17β-estradiol levels were tested for immunoassay interference and showed no effect (data not shown).

TMs, 6 : 2 FTOH, 4 : 2 FTOH, PFBA-PFHpA, and PFNA-PFdoDA were tested in 2–5 experiments in triplicate reactions. Test substances were tested at a maximum concentration of 50 μM and TMs were tested at a maximum concentration of 0.25% of original material across seven twofold dilutions. Vehicle concentrations were kept constant within each cell plate. Testosterone and 17β-estradiol levels were quantified in cell media from experiments with PFBA-PFHpA, 6 : 2 FTOH, and 4 : 2 FTOH exposure, whereas testosterone, 17β-estradiol, as well as progesterone were quantified for PFNA-PFdoDA and the TMs.

Reporter gene assays

The ER, androgen receptor (AR), PPARα, and PPARγ reporter gene assays were conducted as previously described (Vinggaard *et al.*, 2002; Taxvig *et al.*, 2012; Rosenmai *et al.*, 2014). The glucocorticoid receptor (GR) CALUX reporter gene assay was constructed and performed at BioDetection Systems essentially as described previously (Piersma *et al.*, 2013). All assays used luciferase as the reporter.

The ER reporter gene assay was conducted in stably transfected human ovarian adenocarcinoma cell-line ($BG1Luc4E_2$) (Michael Denison, University of California, USA) cultured in RPMI 1649 medium (Life Technology) supplemented with 0.9% pen/strep (Life Technology) and 8% fetal bovine serum (FBS) (Life Technology) until ~72 h before the experiment, where cells were transferred to the DMEM medium (Life Technology) supplemented with 110 mg/mL sodium pyruvate (Fisher Scientific, Hvidovre, Denmark), 1.9% L-glutamine (Fisher Scientific), 0.9% pen/strep (Life Technology), and 4.5% charcoal-treated FBS (Biological Industries, Cromwell, CT, USA). The experiment was initiated by seeding cells at a density of 4×10^4 cells/well in 96-well plates. After ~48 h cells were exposed to test compounds for ~22 h. 17β-estradiol (Steraloids, Newport, RI, USA) was used as a positive control and was tested in concentrations ranging from 3.6×10^{-13} to 3.7×10^{-10} M. Cell viability was assessed

visually as previously described (Rosenmai *et al.*, 2014) or by adding resazurin for 3 h followed by fluorescence measurement.

The AR reporter gene assay was conducted in a Chinese hamster ovary cell-line (ATCC), cultured in DMEM/F12 medium (Life Technologies), supplemented with 10% FBS (Life Technologies), and 1% pen/strep/fungi (Thermo Fisher Scientific, Hvidovre, Denmark). Cell were seeded at a density of 7000 cells/well in 96-well plates in the same medium as above, but now with 10% charcoal-treated FBS (Biological Industries). After ~24 h, cells were transiently transfected with receptors, pSVAR0 (antagonist mode) or pSVAR13 (toxicity) and the reporter gene, MMTV-LUC (Gifts from Albert Brinkmann, Erasmus University, Rotterdam, The Netherlands). Total plasmid concentration was 75 ng/well of 2 : 100 or 1 : 100 for antagonist and toxicity mode respectively. Transfection was conducted with 0.3 μL Fugene/well (Roche, Hvidovre, Denmark) 5 h prior to exposure. R1881 (Perkin Elmer) and hydroxy-flutamide (Toronto Research Chemicals, Ontario, Canada) were used as positive controls and were tested in concentrations ranging from 0.0012 to 2.7 nM and 1 to 5000 nM respectively, in all experiments. Cells were exposed to test compounds for ~19 h. Testing of antagonistic effects of test compounds was done in concert with 0.03 nM or 0.1 nM R1881.

The PPARα and PPARγ reporter gene assay was conducted in NIH-3T3 cells transiently transfected with plasmids, expressing the ligand-binding domain of murine PPARα or PPARγ as well as a plasmid containing the upstream-activating sequence (UAS) (Gifts from Professor Susanne Mandrup, University of Southern Denmark). Cells were grown in DMEM/F12 medium (Life Technologies) supplemented with 10% charcoal-treated FBS (Sigma-Aldrich) and 1% pen/strep/fungi (Thermo Fisher Scientific). Cells were seeded at a density of 7000 cell/well in 96-well plates. After 20 h, transfection was performed with 0.45 μL/well Fugene (Roche) and 75 ng cDNA/well of 1 : 2 and 1 : 1 for PPARα:UAS and PPARγ:UAS respectively. The solution was added to the plates and after 5 h incubation the cells were exposed to test compounds for 22 h. Finally luciferase activity was measured. Positive controls were rosiglitazone (Sigma-Aldrich) and WY 14,643 (Sigma-Aldrich) for PPARγ and PPARα respectively. Rosiglitazone and WY 14,643 were tested in concentrations of 0.01–100 μM. Cytotoxicity was checked in parallel cells transfected with pCMV-luciferase construct, using the same transfection procedure as for the receptor constructs.

The GR-CALUX reporter gene assay was performed in a stably transfected human U2OS osteosarcoma cell-line cultured in DMEM (InVitrogen, Taastrup, Denmark) supplemented with 7.5% FBS (InVitrogen), 1× non-essential amino acids (InVitrogen) and 10 U/mL penicillin and 10 g/mL streptomycin (InVitrogen). Experiments were conducted in DMEM without phenol red (InVitrogen) supplemented with 5% dextran-coated charcoal-stripped fetal calf serum, and amino acids and antibiotics as above. Cells were seeded in 384 well plates at a density of 3×10^3 cells/well. After 24 h cells were exposed to test compounds for another 24 h. Dexamethasone was used as a positive control tested in the range 0.000015–50 nM in all experiments. No cell toxicity measurements were performed in this assay.

All test substances and TMs were tested by ER, PPARα and PPARγ reporter gene assays. TMs, 6 : 2 FTOH, 4 : 2 FTOH, and all PFCAs excluding PFOA, were tested in the AR reporter gene assay and PFOA-PFdoDA were tested in the GR CALUX reporter gene assay.

All assays were performed in 2–6 replicate reactions across 6–10 concentrations and repeated 2–7 times, except 10 : 2 diPAP and 8 : 2 triPAP, which were only tested once for PPARα and PPARγ activity. The maximum tested concentration for TM1 and TM2 was 0.25% of original material and 100 μM for all PFCAs and FTOHs. The maximum tested concentration for PAPs was 50 μM, except in the ER reporter gene assay in which it was 100 μM. A 1 mg/μL ethanol solution of TM3 was prepared, which was diluted by a factor 400 for the tested maximum concentration in the respective assays. Vehicle concentrations were constant for the compounds within each experiment in the AR, PPARα, PPARγ and the GR CALUX reporter gene assay. In the ER reporter gene assay, the vehicle concentration was ≤0.25% in all wells, except for PAPs for which vehicle concentration was 0.5%, but only at the maximum tested concentration.

Ex vivo rat fetal testis steroidogenesis

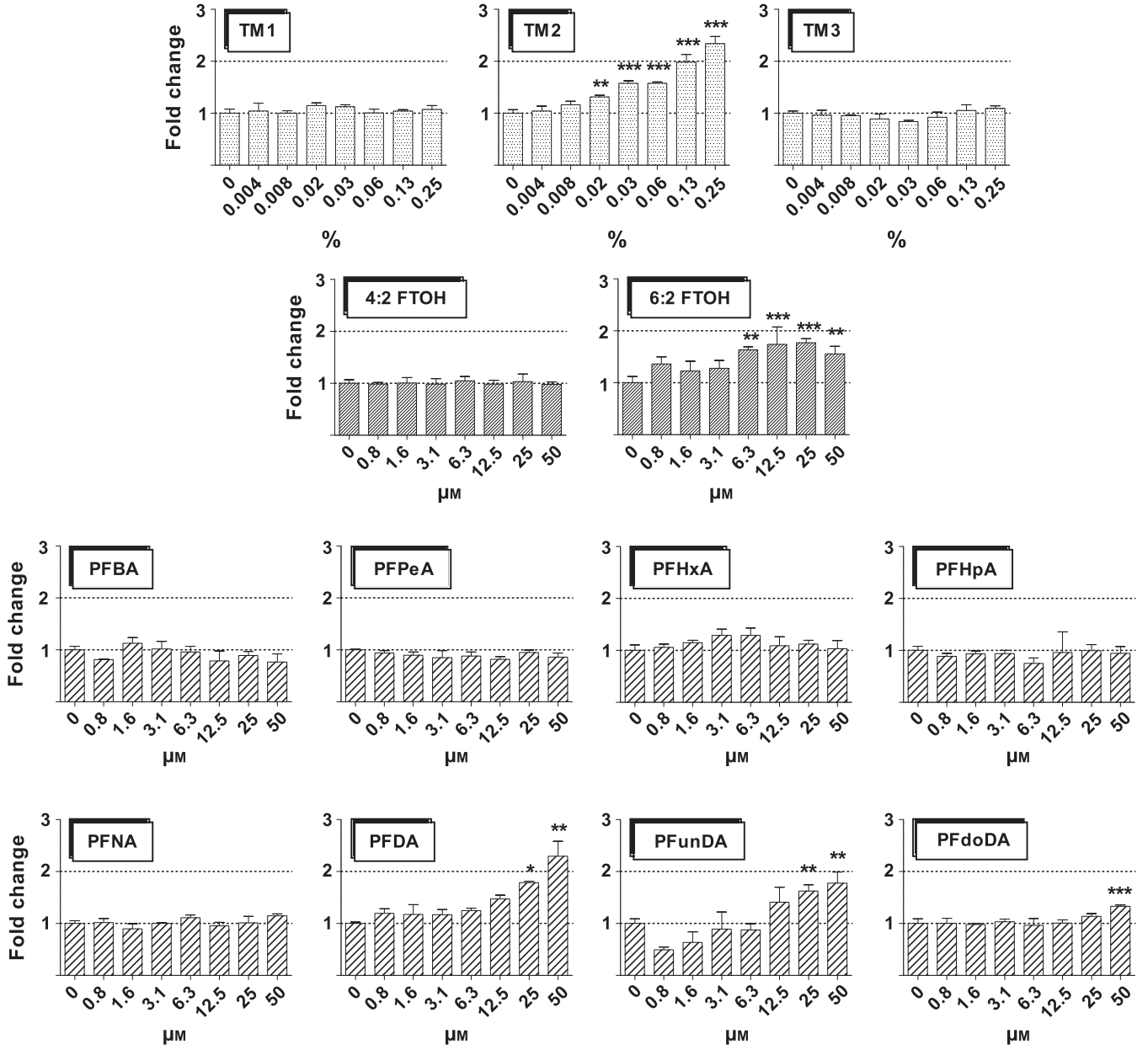
8 : 2 diPAP, 8 : 2 monoPAP and 8 : 2 FTOH, previously reported to affect testosterone levels in the H295R assay (Rosenmai *et al.*, 2013), were analysed using an ex vivo rat fetal testis culture system (FEGA) (Lassurguère *et al.*, 2003; Chauvigné *et al.*, 2009). Animals were housed in a licensed facility in accordance with the French Ministry of Agriculture (agreement # C 35-238-19). Animal experiments were carried out in accordance with the ethical guidelines stipulated by the NIH Guide for Care and Use of the Laboratory Animals and were approved by the Rennes Animal Experimentation Ethics Committee (#R-2012-CCh-01). In brief, fetuses were collected from pregnant Sprague–Dawley rats 14.5 days *post-coitum* and testes dissected out in medium under the microscope. Individual testes were placed on a filter and floated atop of culture medium with test substances for 72 h. Medium was changed at 24 and 48 h, and collected at 24, 48 and 72 h for hormone analysis. 3–5 independent experiments were conducted in 4–8 repeated reactions in six twofold dilutions with a maximum concentration of 50 μM and a constant vehicle concentration. Hormone analysis was conducted using RIA assays according to manufacturer's protocol (Immunotech, Quebec, Canada, Beckman Coulter, Copenhagen, Denmark) or by High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS/MS) (Mortensen & Pedersen, 2007; Rosenmai *et al.*, 2013).

Data processing and statistical analysis

All statistical analyses for in vitro measurements were performed on normalized data from individual experiments, except a few instances where normalized means were pooled. Residuals to normalized mean within each exposure group were pooled and tested for normal distribution by use of the D'Agostino Pearson's Omnibus test. If pooled residuals were normally distributed, a one-way ANOVA (post-test Dunnett) was performed. A Kruskal–Wallis (post-test Dunn) was performed, if pooled residuals were not normally distributed.

If the post-test led to a significant dose-dependent effect in the majority of experiments in the GR CALUX, PPARα, PPARγ, AR and ER reporter gene assays, this was perceived as an effect. If statistically significant dose-dependent cytotoxicity was observed, these exposure groups were not considered further. In the steroidogenesis assay, effects were reported if the majority of experiments showed significant changes in the post-test in response to increasing non-cytotoxic exposure concentrations.

Figure 2 17β-estradiol levels following exposure to TMs, FTOHs, and PFCAs in the steroidogenesis assay. TM2, 6 : 2 FTOH, and PFDA-PFdoDA increased 17β-estradiol levels, and TM1, TM3, 4 : 2 FTOH, PFBA-PFHpA, and PFNA did not. Fold change (±SD) as a function of percent (%) original material provided for TMs and exposure concentration (μM) for FTOH and PFCAs. Graphs depicts one representative experiment. The markings *, **, and *** correspond to *p*-values of <0.05, <0.01, and <0.001.



Hormone measurements from the ex vivo testis assay were statistically analysed according to the same criteria as that for in vitro data on absolute concentrations from single experiments and pooled normalized means from independent experiments.

All data processing and statistical analyses were performed in GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA).

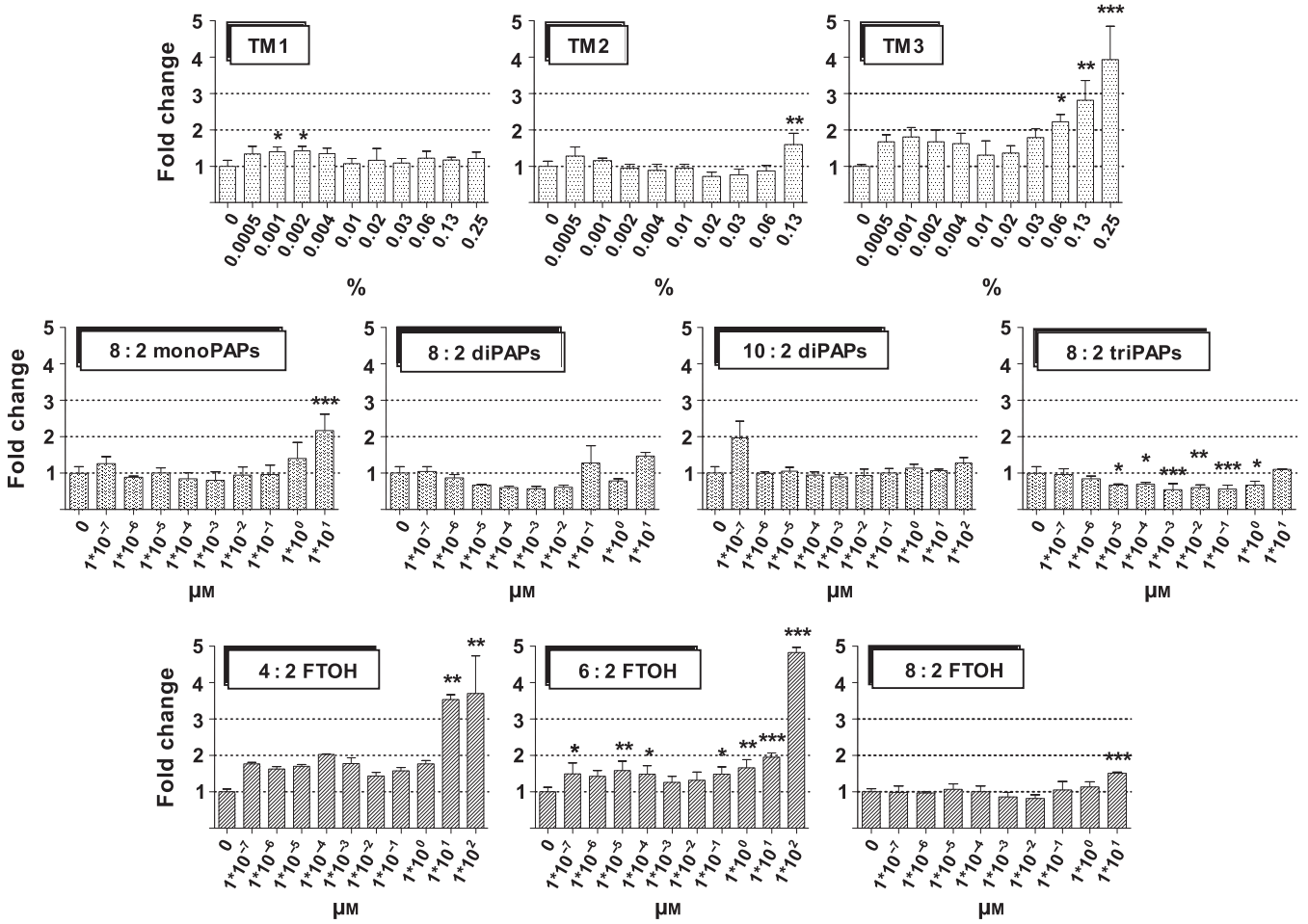
RESULTS

Effects on steroidogenesis

Basal hormone production in the H295R steroidogenesis assay was 521–1940 pg/mL, 365–646 pg/mL, and 8–61 pg/mL for testosterone, progesterone and 17β-estradiol, respectively. Here 17β-estradiol and testosterone were induced by forskolin by 11.6–54.6 and 1.6–11.5-fold change compared to the control respectively, and inhibited with 0.1–0.7 and 0.004–0.04-fold change by prochloraz (data not shown) in the experiments presented in Fig. 2, showing responsiveness of cells to known inducers and inhibitors of steroidogenesis.

As PAPs, 8 : 2 FTOH and PFOA had previously been found to affect in vitro steroidogenesis in our laboratory, we decided to investigate a broader panel of PFAS in this assay. Exposure to TM2, 6 : 2 FTOH, PFDA, PFunDA, and PFdoDA caused a significant increase in 17β-estradiol levels (Fig. 2). PFDA-PFdoDA showed effects at concentration ≥25 μM, whereas 6 : 2 FTOH exhibited effects at 6.3 μM. TM2, 6 : 2 FTOH, PFDA, and

Figure 3 Estrogen receptor activity following exposure to TMs, PAPs, and FTOHs as measured in the ER reporter gene assay. TM2, TM3, 8 : 2 monoPAP, and 4 : 2–8 : 2 FTOH increased ER activity, whereas TM1, 8 : 2 diPAP, and 10 : 2 diPAP did not, and 8 : 2 triPAP led to an apparent decrease. Fold change (±SD) as a function of percent (%) original material provided for TMs and exposure concentration (μM) for FTOHs and PAPs. Graphs depicts one representative experiment. The markings *, **, and *** correspond to *p*-values of <0.05, <0.01, and <0.001.



PFunDA caused increases in response of around 100%, whereas PFdoDA was less efficacious. For PFDA-PFdoDA, the increase was accompanied by an increased response in the MTT assay in one or all experiments, however this was not observed in the fluorescamin assay, and was thus not considered a result of increased cell number. MTT data for experiments presented in Fig. 2 are shown in Figure S4. PFBA-PFHpA, PFNA, TM1, TM3 and 4 : 2 FTOH did not affect 17β-estradiol levels (Fig. 2). None of the test substances affected testosterone or progesterone levels (data not shown).

Effects on rat fetal testis steroidogenesis ex vivo

8 : 2 FTOH, 8 : 2 monoPAP and 8 : 2 diPAP did not significantly affect testosterone levels in the FEGA at any of the time points measured (Figure S1). In support of these RIA measurements, testosterone and androstenedione levels did not change significantly with exposure to 8 : 2 FTOH, 8 : 2 monoPAP, and 8 : 2 diPAP when measured by HPLC-MS/MS (data not shown).

ER activity

17β-estradiol-induced ER activity with 4.9–7.7-fold change compared to vehicle controls for the experiments presented in Fig. 3.

TM2, TM3, 8 : 2 monoPAP, 4 : 2 FTOH, 6 : 2 FTOH, and 8 : 2 FTOH led to increased ER activity with TM3, 4 : 2 FTOH, and 6 : 2 FTOH showing the largest change in response (200–400%) (Table 2, Fig. 3). PFOA and PFNA led to an apparent increase in ER activation at high exposure concentrations, however an increased response was also observed in one of two resazurin cell viability experiments at the same concentrations (data not shown). PFBA-PFHpA and PFDA-PFdoDA did not affect ER activity (data not shown) neither did TM1, 8 : 2 diPAP and 10 : 2 diPAP exposure, whereas 8 : 2 triPAP led to an apparent decreased response (Fig. 3).

AR antagonism

R1881 exposure led to a maximum induction of AR activity of 15.6–74.8-fold change, whereas OHF reduced the AR-mediated activity by 0.1–0.3-fold change compared to the vehicle controls in the experiments presented in Figure S2. None of the test substances led to effects on AR activity (Figure S2) without concomitant cell toxicity at the same concentrations (Figure S5).

PPARα and PPARγ activity

Rosiglitazone led to increased PPARγ activity with 24.8–74.4-fold change, whereas WY 14,643 increased PPARα activity with

Table 2 Qualitative effects of PFAS and technical mixtures (TMs) on progesterone (P), testosterone (T) and 17β-estradiol levels in the steroidogenesis assay (H295R) and the ex vivo fetal rat model (FEGA)

	H295R			FEGA	hER	PPARα	PPARγ	AR	GR
	P	T	E	T					
TM1[f]	–	–	–		–	–	–	–	
TM2[f]	–	–	↑		↑	–	–	–	
TM3[g]	–	–	–		↑	–	–	–	
8 : 2 triPAP	↓[a]	↓[a]	↑[a,b]		–	–	–	–[a]	
10 : 2 diPAP	↓[a]	–[a]	↑[a,b]		–	–	–	–[a]	
8 : 2 diPAP	↓[a]	↓[a]	↑[a,b]	–	–	–	–	–[a]	
8 : 2 monoPAP	↓[a]	↓[a]	↑[a,b]	–	↑	–	–	–[a]	
8 : 2 FTOH	↓[a]	↓[a]	↑[a,b]	–	↑	–	–	–[a]	
6 : 2 FTOH		–	↑		↑	–	–	–	
4 : 2 FTOH		–	–		↑	–	–	–	
PFdoDA[g]	–	–	↑[c]		–	↑	↑	–	–
PFunDA	–	–	↑[c]		–	↑	↑	–	–
PFDA	–	–	↑[c]		–	↑	↑	–	–
PFNA	–	–	–		(↑)[e]	↑	↑	–	–
PFOA	–[a]	–[a]	↓↑[a,b,d]		(↑)[e]	↑	↑	–[a]	–
PFHpA		–	–		–	↑	↑	–	
PFHxA		–	–		–	↑	↑	–	
PFPeA		–	–		–	↑	↑	–	
PFBA		–	–		–	↑	–	–	

Results from reporter gene assays including the human estrogen receptor (hER), androgen receptor (AR), glucocorticoid receptor (GR), and peroxisome proliferator-activated receptor α and γ (PPARα and PPARγ) are presented. Results are based on statistically significant effects observed in the majority of independent experiments. Increased responses are indicated as (↑, light grey), decreased responses as (↓, dark grey), no effect with a dash (–), and an empty field means not tested/measured. (a) results from Rosenmai *et al.* (2013), (b) effects reported for 17β-estradiol and/or estrone, (c) response accompanied by increased MTT response, but not in the fluorescamine assay, (d) an U-shaped concentration–response curve was reported with a decrease in E2 levels at lower concentrations and an increase at higher concentrations, (e) increased responses at high exposure concentrations accompanied by increases in the resazurin cell viability assay, (f) stock solution dissolved in water, (g) stock solution dissolved in ethanol. All other stock solutions were made in dimethyl sulfoxide.

2.3–6.9-fold change compared to the vehicle controls in the experiments presented in Fig. 4.

All PFCAs led to increased PPARα and PPARγ activity from exposure concentrations of 30 μM or 100 μM, except for PFBA, which did not cause any change in PPARγ activity (Fig. 4). The increases in response on PPARα and PPARγ ranged between 37 and 139%. The FTOHs, PAPs and TMs did not cause any significant change in activity of the PPARs (data not shown). None of the test compounds affected cell viability in a dose-dependent manner (Figure S6).

GR CALUX reporter gene activity

Dexamethasone increased GR activity by 75.2 and 81.5-fold change compared to the vehicle controls in the two experiments conducted, however the PFOA-PFdoDA did not lead to increased activity of the receptor (Figure S3).

DISCUSSION

We have analysed the endocrine activity of a number of PFAS, with particular focus on; (i) substances used in food packaging materials (PAPs), (ii) short-chain PFAS occurring as metabolic products of the functional coating ingredient or as impurities in some TMs (FTOH and PFCAs), and (iii) three commercially available TMs containing PFAS of unknown chemical composition. As summarized in Table 2, the most prominent effect was estrogenic activity as well as PPARα and PPARγ activity. The potency and/or efficacy of PFAS on these endpoints were generally lower than the positive controls. Nevertheless, humans will be exposed to other PFAS and other compounds with similar activities, and may thus combine to elicit an effect.

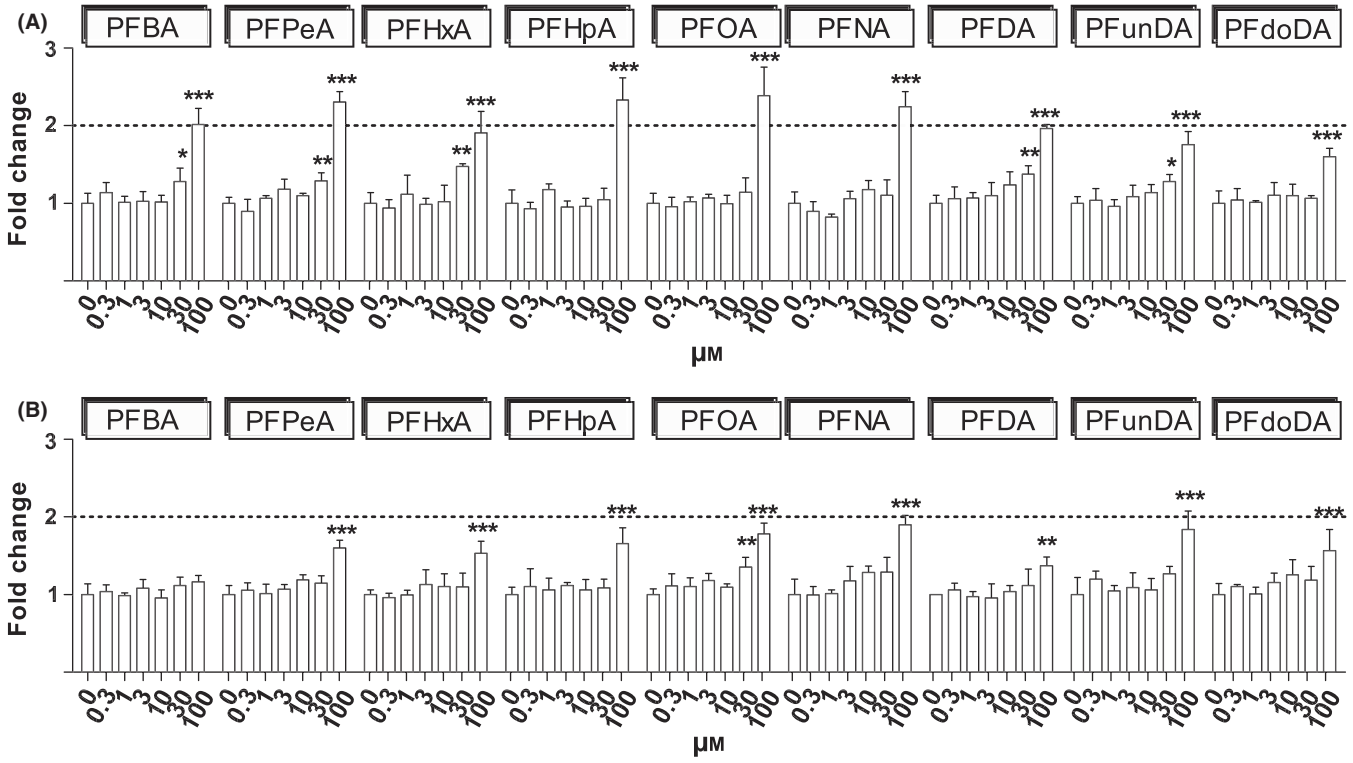
Some long-chain PFCAs are reported to exhibit estrogenic and antiandrogenic activities. Regarding estrogenic activity, PFNA exposure can increase serum 17β-estradiol levels in vivo (Feng *et al.*, 2009) and in vitro (Kraugerud *et al.*, 2011). We observed elevated 17β-estradiol levels following PFDA-PFdoDA, but not PFNA exposure. These increases could be due to interferences with aromatase activity or metabolism of estrogen. Taken together, the estrogenic activity of long-chain PFCAs is of concern, not least because some of these substances are persistent in humans and that in utero exposure to estrogenic compounds is associated with adverse health effects, including breast cancer (Soto *et al.*, 2013) and altered male fetal reproductive development (Toppari *et al.*, 1996).

Regarding anti-androgenicity, PFOA-PFDA did not affect AR activity as reported previously (Kjeldsen & Bonefeld-Jorgensen, 2013). PFDA and PFdoDA can decrease blood testosterone levels in male rats (Bookstaff *et al.*, 1990; Shi *et al.*, 2009), however we observed no effect on testosterone levels in the H295R assay. These deviations between in vitro and in vivo responses indicate that the in vivo effects are not always due to a direct effect on the intracellular steroidogenic pathway, but rather a result of, for instance differences in toxicokinetics or interferences with the hypothalamic–pituitary axis.

8 : 2 diPAP, 8 : 2 monoPAP, and 8 : 2 FTOH can inhibit testosterone synthesis in the H295R assay (Rosenmai *et al.*, 2013). Therefore, we tested these substances on explanted rat fetal testes in a model that previously established anti-androgenic activity of environmental contaminants such as bisphenol A and phthalates (Chauvigné *et al.*, 2009; Maamar *et al.*, 2015). We observed no significant effect on secreted testosterone levels following 3 days of exposure. The reason for this discrepancy between assays is unclear, but could be because of differences between a cell-line and an intact organ, or human adrenal vs. rat Leydig cell steroidogenesis. Furthermore, no serum was added to the medium in the FEGA assay, which may have affected the tissue bioavailability of PFAS, as protein can serve as a 'carrier' for a wide range of perfluoroalkyl acids, including carboxylates and sulphonates (Bischel *et al.*, 2010, 2011), thereby transporting PFAS to the tissue and limiting non-specific binding to plastics, etc. Additional studies on the potential anti-androgenic effect of these substances are therefore warranted, particularly because an association is suggested between in utero exposure to antiandrogenic compounds and male reproductive abnormalities (Skakkebaek, 2002).

Even though the industry is changing from long-chain to short-chain chemistry (Scheringer *et al.*, 2014), knowledge about their potential endocrine activities remain limited. Thus, we also included their intermediate metabolites 4 : 2 FTOH-8 : 2 FTOH and the final metabolites PFBA-PFHpA. The estrogenic activity of 6 : 2 and 8 : 2 FTOH has been described in MCF-7 breast cancer cells (Maras *et al.*, 2006). These substances also increased plasma 17β-estradiol in zebrafish (Liu *et al.*, 2009, 2010), which we previously confirmed in vitro for 8 : 2 FTOH (Rosenmai *et al.*, 2013). Additionally, 6 : 2 FTOH increased testosterone levels in zebrafish (Liu *et al.*, 2009), although we did not observe elevated testosterone in this study. Nevertheless, our new data

Figure 4 Peroxisome proliferator-activated receptor α (A) and γ (B) activity following exposure to PFBA-PFdoDA in the PPARα and PPARγ reporter gene assays. All tested PFCAs increased PPARα and PPARγ activity, except PFBA which did not affect PPARγ. Fold change (±SD) as a function of exposure concentration (μM). Graphs depicts one representative experiment, except for PFDA in the PPARγ reporter gene assay, which depicts pooled means of independent experiments. The markings *, **, and *** correspond to *p*-values of <0.05, <0.01, and <0.001.



corroborates previous findings with respect to estrogenic activity, now including 4 : 2 and 6 : 2 FTOH (Table 2).

PFCAs with chain lengths 4–7 could be less hazardous with respect to estrogenic activity than those with long chains, as none of these affected 17β-estradiol levels. In contrast, their short chain precursors, 4 : 2 and 6 : 2 FTOH that ultimately can be metabolized into short-chain PFCAs exhibited estrogenic activity as described above. Short-chain PFCAs caused PPARα and PPARγ activation, except PFBA which had no effect on PPARγ. This is in line with previous reports (Buhrke *et al.*, 2013; Zhang *et al.*, 2014). The potency and efficacy of short-chain PFCAs was in the same range as long-chain PFCAs and equally potent on both receptors under our experimental conditions. PFOA can induce tumour development in testes, pancreas and liver in rats and a triad of tumours associated with exposure to some PPARα agonists (Lau, 2012). Furthermore, as PPARγ is involved in adipocyte differentiation (Ferré, 2004), PFAS affecting this receptor may be involved in development of obesity. Therefore, this and other studies points towards further studies on short-chain PFAS to be conducted to ascertain that these are safe to use.

The three tested TMs are all commercially available and intended for coating of food packaging of paper and board. Migration of PFAS in commercial TMs, their degradation products, and impurities to food has been measured (Begley *et al.*, 2005, 2008; Tittlemier *et al.*, 2006; Trier, 2012; Gebbink *et al.*, 2013). The general type of fluorocarbon and percentage of fluorocarbon content are listed on the product datasheets. However, information on specific fluorinated chemical structures and impurities are not readily available. The PFAS monomer and oligomer structures in TM1 have been characterized (Dimzon *et al.*, 2015), but not in TM2 and TM3, where only the general chemical composition given in the technical data sheets are known. This in itself limits our ability to conclude on active substances, but this also pose challenges with respect to choosing suitable solvents for all components of the mixtures. Thus, interpretation of results for TMs should be carried out with caution, however, the results show that some component(s) of TM2 and TM3 exhibit estrogenic activities. Whether these components are fluorinated is unknown, although we are tempted to suggest this as we found the same response for some pure PFAS. Furthermore, data are relevant as we used the solvents recommended by the manufacturers of the TMs used for food packaging material, meaning that the PFAS solutions applied for the cell studies and for production of the food packaging material are likely the same.

PAPs can metabolize in vivo into the final metabolites PFCAs through the intermediate metabolites FTOHs (D'eon & Mabury, 2007, 2011; Butt *et al.*, 2014). Thus, our study allowed comparison of effects across interconnected groups of substances and assays. In a previous study, we showed that PAPs affect steroidogenesis (Rosenmai *et al.*, 2013) and we can now include effects of short-chain FTOHs and long-chain PFCAs (Table 2). In general, PAPs and 8 : 2 FTOH suppressed androgen and progesterone levels (Rosenmai *et al.*, 2013), whereas neither the short-chain FTOHs nor the PFCAs did in the present study. Also, PAPs, 8 : 2 FTOH, 6 : 2 FTOH and long-chain PFCAs elevated 17β-estradiol levels, whereas neither 4 : 2 FTOH nor short-chain PFCAs did. This strongly suggest that structural differences such as the size of the molecule, presence of a hydrocarbon segment, the functional head group and the chain length are central to the

effect on steroidogenesis. Additionally, all FTOHs and 8 : 2 monoPAP-activated hER (Table 2), whereas di- and tri-alkylated PAP as well as most PFCAs did not. Finally, all PFCAs-activated PPARα and PPARγ with similar potencies, except PPARγ activation by PFBA, whereas none of the other compounds elicited activities in the PPAR assays, findings which are in line with previous reports (Buhrke *et al.*, 2013; Zhang *et al.*, 2014). Again this suggests that structural characteristics such as fluorination, hydrocarbon segment and functional head group, play a role for the ER, PPARα and PPARγ activity. If however, the test compounds were metabolized in the cellular assay, for instance by PAPs metabolizing to PFCAs, these deductions about structural activities may not hold true. Nevertheless, the ER activity, and effects on progesterone and testosterone levels were generally observed for PAPs and/or FTOHs, but generally not for their metabolites PFCAs, meaning that these had not been completely metabolized into PFCAs. Furthermore, the lack of PPAR activities following exposure to PAPs and FTOHs suggest that these were not transformed into PFCAs, as these do exhibit activity.

In conclusion, we have shown that PFAS have estrogenic activity and that parent substances used in paper packaging for food have different biomolecular effects than their metabolites. Some TMs showed estrogenic activities, but the short-chain PFCAs did not. Furthermore, we showed that the final metabolites (PFCA) can activate PPARα and PPARγ with a similar potency, whereas their parent substances cannot. Notably, we observed little effects on anti-/androgenic measurements, but this does not exclude PFAS from affecting the male reproductive health, as estrogens also play important roles for male reproductive development and function (Rouiller-Fabre *et al.*, 2015). Collectively, our data suggests that the degree of fluorination, the degree of alkylation, the length of the fluorinated chain, the size of the molecule and the functional head group, all play central roles in determining the effects elicited. Future studies should focus on investigating the molecular mechanisms behind the different activities as well as the effects of short-chain PFAS and other FTOH-derived TMs to assure that these are safe to use for widespread applications.

SUPPLEMENTARY DATA

Supplementary data is available online for the FEGA assay, the AR reporter gene assay, the GR CALUX reporter gene assay as well as the cell viability assays.

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REFERENCES

Begley TH, White K, Honigfort P, Twaroski ML, Neches R & Walker RA. (2005) Perfluorochemicals: potential sources of and migration from food packaging. *Food Addit Contam* 22, 1023–1031.

Begley TH, Hsu W, Noonan G & Diachenko G. (2008) Migration of fluorochemical paper additives from food-contact paper into foods and food simulants. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 25, 384–390.

BfR (2015) Standard XXXVI. Paper and board for food contact. Federal Institute for Risk Assessment, Germany. Available at: https://bfr.ble.de/kse/faces/resources/pdf/360-english.pdf Published 010715. Accessed on 28 January 2016.

Biegel LB, Liu RCM, Hurt ME & Cook JC. (1995) Effects of ammonium perfluorooctanoate on Leydig-cell function: in-vitro, in-vivo, and ex-vivo studies. *Toxicol Appl Pharmacol* 134, 18–25.

Biegel LB, Hurtt ME, Frame SR, O'Connor JC & Cook JC. (2001) Mechanisms of extrahepatic tumor induction by peroxisome proliferators in male CD rats. *Toxicol Sci* 60, 44–55.

Bischel HN, MacManus-Spencer LA & Luthy RG. (2010) Noncovalent interaction of long-chain perfluoroalkyl acids with serum albumin. *Environ Sci Technol* 44, 5263–5269.

Bischel HN, MacManus-Spencer LA, Zhang C & Luthy RG. (2011) Strong association of short-chain perfluoroalkyl acids with serum albumin and investigation of binding mechanisms. *Environ Toxicol Chem* 30, 2423–2430.

Bookstaff RC, Moore RW, Ingall GB & Peterson RE. (1990) Androgenic deficiency in male rats treated with perfluorodecanoic acid. *Toxicol Appl Pharmacol* 104, 322–333.

Borchers A, Teuber SS, Keen CL & Gershwin ME. (2010) Food safety. *Clin Rev Allergy Immunol* 39, 95–141.

Buhrke T, Kibellus A & Lampen A. (2013) In vitro toxicological characterization of perfluorinated carboxylic acids with different carbon chain lengths. *Toxicol Lett* 218, 97–104.

Butt CM, Muir DCG & Mabury SA. (2014) Biotransformation pathways of fluorotelomer-based polyfluoroalkyl substances: a review. *Environ Toxicol Chem* 33, 243–267.

Calafat AM, Wong LY, Kuklenyik Z, Reidy JA & Needham LL. (2007) Polyfluoroalkyl chemicals in the US population: data from the national health and nutrition examination survey (NHANES) 2003-2004 and comparisons with NHANES 1999-2000. *Environ Health Perspect* 115, 1596–1602.

Chauvigné F, Menuet A, Lesné L, Chagnon MC, Chevrier C, Regnier JF, Angerer J & Jégou B. (2009) Time- and dose-related effects of di-(2-ethylhexyl) phthalate and its main metabolites on the function of the rat fetal testis in vitro. *Environ Health Perspect* 117, 515–521.

Cook JC, Murray SM, Frame SR & Hurtt ME. (1992) Induction of Leydig cell adenomas by ammonium perfluorooctanoate: a possible endocrine-related mechanism. *Toxicol Appl Pharmacol* 113, 209–217.

D'eon JC & Mabury SA (2007) Production of perfluorinated carboxylic acids (PFCAs) from the biotransformation of polyfluoroalkyl phosphate surfactants (PAPS): exploring routes of human contamination. *Environ Sci Technol* 41, 4799–4805.

D'eon JC & Mabury SA (2011) Exploring indirect sources of human exposure to perfluoroalkyl carboxylates (PFCAs): evaluating uptake, elimination, and biotransformation of polyfluoroalkyl phosphate esters (PAPs) in the rat. *Environ Health Perspect* 119, 344–350.

D'eon JC, Crozier PW, Furdui VI, Reiner EJ, Libelo EL & Mabury SA (2009) Observation of a commercial fluorinated material, the polyfluoroalkyl phosphoric acid diesters, in human sera, wastewater treatment plant sludge, and paper fibers. *Environ Sci Technol* 43, 4589–4594.

Dimzon IKD, Trier X, Frömel T, Helmus R, Knepper TP & de Voogt P (2015) High Resolution Mass Spectrometry of Polyfluorinated Polyether-based Formulation. *J Am Soc Mass Spectrom* 27, 309–318. Published online. doi:10.1007/s13361-015-1269-9.

Feng Y, Shi Z, Fang X, Xu M & Dai J. (2009) Perfluorononanoic acid induces apoptosis involving the Fas death receptor signaling pathway in rat testis. *Toxicol Lett* 190, 224–230.

Ferré P. (2004) The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. *Diabetes* 53, S43–S50.

Gebbink WA, Ullah S, Sandblom O & Berger U. (2013) Polyfluoroalkyl phosphate esters and perfluoroalkyl carboxylic acids in target food samples and packaging - method development and screening. *Environ Sci Pollut Res Int* 20, 7949–7958.

Houde M, Martin JW, Letcher RJ, Solomon KR & Muir DCG. (2006) Biological monitoring of polyfluoroalkyl substances: a review. *Environ Sci Technol* 40, 3463–3473.

Kim SK, Lee KT, Kang CS, Tao L, Kannan K, Kim KR, Kim CK, Lee JS, Park PS, Yoo YW, Ha JY, Shin YS & Lee JH. (2011) Distribution of perfluorochemicals between sera and milk from the same mothers and implications for prenatal and postnatal exposures. *Environ Pollut* 159, 169–174.

Kissa E. (2001) *Fluorinated Surfactants and Repellents*, 2nd edn. Marcel Dekker, New York.

Kjeldsen LS & Bonefeld-Jorgensen EC. (2013) Perfluorinated compounds affect the function of sex hormone receptors. *Environ Sci Pollut Res Int* 20, 8031–8044.

Kraugerud M, Zimmer KE, Ropstad E & Verhaegen S. (2011) Perfluorinated compounds differentially affect steroidogenesis and viability in the human adrenocortical carcinoma (H295R) in vitro cell assay. *Toxicol Lett* 205, 62–68.

Kubwabo C, Kosarac I & Lalonde K. (2013) Determination of selected perfluorinated compounds and polyfluoroalkyl phosphate surfactants in human milk. *Chemosphere* 91, 771–777.

Lassurguère J, Livera G, Habert R & Jégou B. (2003) Time- and dose-related effects of estradiol and diethylstilbestrol on the morphology and function of the fetal rat testis in culture. *Toxicol Sci* 73, 160–169.

Lau C. (2012) Perfluorinated compounds. In *Molecular, Clinical and Environmental Toxicology* (ed A Luch), Vol 3, pp. 47–86. Springer, Heidelberg, Germany.

Liu C, Yu L, Deng J, Lam PKS, Wu RSS & Zhou B. (2009) Waterborne exposure to fluorotelomer alcohol 6:2 FTOH alters plasma sex hormone and gene transcription in the hypothalamic-pituitary-gonadal (HPG) axis of zebrafish. *Aquat Toxicol* 93, 131–137.

Liu C, Deng J, Yu L, Ramesh M & Zhou B. (2010) Endocrine disruption and reproductive impairment in zebrafish by exposure to 8:2 fluorotelomer alcohol. *Aquat Toxicol* 96, 70–76.

Maamar MB, Lesné L, Desdoits-Lethimonier C, Coiffec I, Lassurguère J, Lavoué V, Deceuninck Y, Antignac J, Bizec B, Perdu E, Zalko D, Pineau C, Chevrier C, Dejucq-Rainsford N, Mazaud-Guittot S & Jégou B. (2015) An investigation of the endocrine-disruptive effects of bisphenol A in human and rat fetal testes. *PLoS ONE* 10, 1–18.

Maras M, Vanparys C, Muylle F, Robbens J, Berger U, Barber JL, Blust R & De Coen W. (2006) Estrogen-like properties of fluorotelomer alcohols as revealed by MCF-7 breast cancer cell proliferation. *Environ Health Perspect* 114, 100–105.

Monroy R, Morrison K, Teo K, Atkinson S, Kubwabo C, Stewart B & Foster WG. (2008) Serum levels of perfluoroalkyl compounds in human maternal and umbilical cord blood samples. *Environ Res* 108, 56–62.

Mortensen SK & Pedersen M. (2007) Confirmatory analysis of acetylgestagens in plasma using liquid chromatography-tandem mass spectrometry. *Anal Chim Acta* 586, 217–222.

Olsen GW, Burris JM, Ehresman DJ, Froehlich JW, Seacat AM, Butenhoff JL & Zobel LR. (2007) Half-life of serum elimination of perfluorooctanesulfonate, perfluorohexanesulfonate, and perfluorooctanoate in retired fluorochemical production workers. *Environ Health Perspect* 115, 1298–1305.

Olsen GW, Lange CC, Ellefson ME, Mair DC, Church TR, Goldberg CL, Herron RM, Medhdizadehkashi Z, Nobiletti JB, Rios JA, Reagen WK & Zobel LR. (2012) Temporal trends of perfluoroalkyl concentrations in American red cross adult blood donors, 2000-2010. *Environ Sci Technol* 46, 6330–6338.

Pérez F, Nadal M, Navarro-Ortega A, Fàbrega F, Domingo JL, Barcelo D & Farré M. (2013) Accumulation of perfluoroalkyl substances in human tissues. *Environ Int* 59, 354–362.

Pérez F, Llorca M, Köck-Schulmeyer M, Škrbić B, Oliveira LS, Martinello KDB, Al-Dhabi NA, Antić I, Farré M & Barceló D. (2014) Assessment of perfluoroalkyl substances in food items at global scale. *Environ Res* 135, 181–189.

Piersma AH, Bosgra S, van Duursen MBM, Hermsen SAB, Jonker LRA, Kroese ED, van der Linden SC, Man H, Roelofs MJ, Schulpen SH, Schwarz M, Uibel F, van Vugt-Lussenburg BM, Westerhout J, Wolterbeek AP & van der Burg B. (2013) Evaluation of an alternative in vitro test battery for detecting reproductive toxicants. *Reprod Toxicol* 38, 53–64.

Prevedouros K, Cousins IT, Buck RC & Korzeniowski SH. (2006) Sources, fate and transport of perfluorocarboxylates. *Environ Sci Technol* 40, 32–44.

Rosenmai AK, Nielsen FK, Pedersen M, Hadrup N, Trier X, Christensen JH & Vinggaard AM. (2013) Fluorochemicals used in food packaging inhibit male sex hormone synthesis. *Toxicol Appl Pharmacol* 266, 132–142.

Rosenmai AK, Dybdahl M, Pedersen M, van Vugt-Lussenburg BMA, Wedebye EB, Taxvig C & Vinggaard AM. (2014) Are structural analogues to bisphenol A safe alternatives? *Toxicol Sci* 139, 35–47.

Rouiller-Fabre V, Guerquin MJ, N'Tumba-Byn T, Muczynski V, Moison D, Tourpin S, Messiaen S, Habert R & Livera G. (2015) Nuclear receptors and endocrine disruptors in fetal and neonatal testes: a gapped landscape. *Front Endocrinol* 6, 1–13.

Scheringer M, Trier X, Cousins IT, de Voogt P, Fletcher T, Wang Z & Webster TF. (2014) Helsingor statement on poly- and perfluorinated alkyl substances (PFASs). *Chemosphere* 114, 337–339.

Shi Z, Ding L, Zhang H, Feng Y, Xu M & Dai J. (2009) Chronic exposure to perfluorododecanoic acid disrupts testicular steroidogenesis and the expression of related genes in male rats. *Toxicol Lett* 188, 192–200.

Shi Z, Zhang H, Ding L, Feng Y, Wang J & Dai J. (2010) Proteomic analysis for testis of rats chronically exposed to perfluorododecanoic acid. *Toxicol Lett* 192, 179–188.

Skakkebaek NE. (2002) Endocrine disrupters and testicular dysgenesis syndrome. *Horm Res* 57, 43–43.

Skakkebaek NE, Rajpert-De Meyts E & Main KM. (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod* 16, 972–978.

So MK, Yamashita N, Taniyasu S, Jiang QT, Giesy JP, Chen KN & Lam PK. (2006) Health risks in infants associated with exposure to perfluorinated compounds in human breast milk from Zhoushan, China. *Environ Sci Technol* 40, 2924–2929.

Soto AM, Brisken C, Schaeberle C & Sonnenschein C. (2013) Does cancer start in the womb? Altered mammary gland development and predisposition to breast cancer due to in utero exposure to endocrine disruptors. *J Mammary Gland Biol Neoplasia* 18, 199–208.

Stahl T, Mattern D & Brunn H. (2011) Toxicology of perfluorinated compounds. *Environ Sci Eur* 23, 1–52.

Taxvig C, Dreisig K, Boberg J, Nellemann C, Schelde AB, Pedersen D, Boergesen M, Mandrup S & Vinggaard AM. (2012) Differential effects of environmental chemicals and food contaminants on adipogenesis, biomarker release and PPARγ activation. *Mol Cell Endocrinol* 361, 106–115.

The Danish Veterinary and Food Administration, Ministry of Environment and Food (2015) Pap og paper – fluorerede stoffer. Available at: http://www.foedevarestyrelsen.dk/Leksikon/Sider/Papir-og-pap.aspx. Published 2015. Updated 180915. Accessed on 23 September 2015.

The European Commission (2004). Regulation (EC) no 1935/2004 of the European Parliament and of the Council of 27 October 2004 on materials and articles intended to come into contact with food and repealing Directives 80/590/EEC and 89/109/EEC. Official Journal of

the European Union. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32004R1935&from=EN. Published 2004. Assessed 121215. Accessed on 12 December 2015.

Tittlemier SA, Pepper K & Edwards L. (2006) Concentrations of pefluorooctanesulfonamides in Canadian total diet study composite food samples collected between 1992 and 2004. *J Agric Food Chem* 54, 8385–8389.

Toppari J, Larsen JC, Christiansen P, Giwercman A, Grandjean P, Guillette LJ Jr, Jégou B, Jensen TK, Jouannet P, Keiding N, Leffers H, McLachlan JA, Meyer O, Müller J, Rajpert-De Meyts E, Scheike T, Sharpe R, Sumpter J & Skakkebaek NE. (1996) Male reproductive health and environmental xenoestrogens. *Environ Health Perspect* 104, 741–803.

Trier X. (2012) Danish Survey (2010-2011) of PFAS migration from food packaging paper and board to food simulants and food. Danish Vet Food Auth.

Trier X, Granby K & Christensen JH. (2011) Polyfluorinated surfactants (PFS) in paper and board coatings for food packaging. *Environ Sci Pollut Res Int* 18, 1108–1120.

US FDA (2015) Indirect Food Additives: Paper and Paperboard Components; Code of Federal Regulations, 21 CFR 176.170. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=176.170. Published 010415. Updated 082115. Accessed on 28 January 2016.

Vinggaard AM, Nellemann C, Dalgaard M, Joergensen EB & Andersen HR. (2002) Antiandrogenic effects in vitro and in vivo of the fungicide prochloraz. *Toxicol Sci* 69, 344–353.

Wolf CJ, Takacs ML, Schmid JE, Lau C & Abbott BD. (2008) Activation of mouse and human peroxisome proliferator-activated receptor alpha by perfluoroalkyl acids of different functional groups and chain lengths. *Toxicol Sci* 106, 162–171.

Wolf CJ, Schmid JE, Lau C & Abbott BD. (2012) Activation of mouse and human peroxisome proliferator-activated receptor-alpha (PPARα) by perfluoroalkyl acids (PFAAs): further investigation of C4-C12 compounds. *Reprod Toxicol* 33, 546–551.

Zhang L, Ren XM, Wan B & Guo LH. (2014) Structure-dependent binding and activation of perfluorinated compounds on human peroxisome proliferator-activated receptor γ. *Toxicol Appl Pharmacol* 279, 275–283.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. PFAS effects on testosterone production in rat fetal testis ex vivo.
Figure S2. PFAS effects on androgen receptor antagonism *in vitro*.
Figure S3. PFCA effects on glucocorticoid receptor agonism *in vitro*.
Figure S4. Cytotoxicity of PFAS in the H295R assay.
Figure S5. Cytotoxicity of PFAS in the AR assay.
Figure S6. Cytotoxicity of PFCA in the PPAR assays.