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Authors

Chen, Hao
Kapidzic, Mirhan
Gantar, Danielle
et al.

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Perfluorooctanoic acid induces transcriptomic alterations in second trimester human cytotrophoblasts

Hao Chen [1], Mirhan Kapidzic,[1] Danielle Gantar,[1] Sena Aksel,[1] Justine Levan,[1] Dimitri P. Abrahamsson,[2] Unurzul Jigmeddagva,[1] Sanah Basrai,[1] Ali San,[1] Stephanie L. Gaw,[1] Tracey J. Woodruff,[2] Susan J. Fisher,[1] Joshua F. Robinson [1,2,*]

[1]Center for Reproductive Sciences, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco (UCSF), San Francisco, California 94143, USA
[2]Program on Reproductive Health and the Environment, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco (UCSF), San Francisco, California 94143, USA

*To whom correspondence should be addressed at Department of Obstetrics, Gynecology, and Reproductive Sciences, Center for Reproductive Sciences, University of California, San Francisco (UCSF), 513 Parnassus Ave, Rm. 1621, San Francisco, CA 94143-0665, USA. E-mail: joshua.robinson@ucsf.edu.

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Abstract

Poly- and perfluroroalkylated substances (PFAS) are a major class of surfactants used in industry applications and consumer products. Despite efforts to reduce the usage of PFAS due to their environmental persistence, compounds such as perfluorooctanoic acid (PFOA) are widely detected in human blood and tissue. Although growing evidence supports that prenatal exposures to PFOA and other PFAS are linked to adverse pregnancy outcomes, the target organs and pathways remain unclear. Recent investigations in mouse and human cell lines suggest that PFAS may impact the placenta and impair trophoblast function. In this study, we investigated the effects of PFOA on cytotoxicity and the transcriptome in cultured second trimester human cytotrophoblasts (CTBs). We show that PFOA significantly reduces viability and induces cell death at 24 h, in a concentration-dependent manner. At subcytotoxic concentrations, PFOA impacted expression of hundreds of genes, including several molecules (*CRH*, *IFIT1*, and *TNFSF10*) linked with lipid metabolism and innate immune response pathways. Furthermore, *in silico* analyses suggested that regulatory factors such as peroxisome proliferator-activated receptor-mediated pathways may be especially important in response to PFOA. In summary, this study provides evidence that PFOA alters primary human CTB viability and gene pathways that could contribute to placental dysfunction and disease.

Keywords: per- and polyfluoroalkyl substances (PFAS); human; placenta; cytotrophoblast; transcriptomics; peroxisome proliferator-activated receptor.

Comprised of a carbon backbone and charged substituents (eg, sulfate or fluorine groups), per- and polyfluoroalkyl substances (PFAS) are synthetic compounds used as surfactants in consumer products (Frömel and Knepper, 2010) and industrial processes (Tabuchi, 2021). Over 600 PFAS are actively used in the United States (U.S. EPA). As a result, many PFAS and their breakdown products (approximately 12 000 unique species [USEPA]) are detected in both indoor and outdoor environments (Viberg and Eriksson, 2011). Warranting concern, 18–80 million Americans drink water contaminated with perfluorooctanoic acid (PFOA) and perfluorooctanesulfonate (PFOS) at combined concentrations above 10 ng/l (Andrews and Naidenko, 2020; Domingo and Nadal, 2019).

Despite gradual voluntary phasing out of PFOA in the marketplace due to its biopersistence and suspected toxicities as a carcinogen, liver, developmental, immune toxicant, and endocrine disruptor (Fenton *et al.*, 2021), PFOA is detected in the overwhelming majority of individuals in the United States (Calafat *et al.*, 2007). Data from the National Health and Nutrition Examination Survey indicate blood levels of PFOA are detected in 99% of U.S. individuals (Woodruff *et al.*, 2011). Median maternal blood levels of PFOA are approximately 1–4 ng/ml (Calafat *et al.*, 2007; CDC, 2022a,b; Kato *et al.*, 2011). PFOA and other PFAS can cross the placenta and expose the developing embryo/fetus to concentrations comparable with those found in maternal blood (Abrahamsson *et al.*, 2022). These compounds bioaccumulate in the placenta and other embryonic/fetal tissues (Hall *et al.*, 2022; Mamsen *et al.*, 2017) with heightened exposures occurring in the second and third trimesters (Mamsen *et al.*, 2019). Although recent estimates suggest that PFOA levels are gradually declining, and alternatives to PFOA are increasing (Gewurtz *et al.*, 2019; Kato *et al.*, 2011), PFOA exposures remain of concern due to their persistence, ubiquity, and growing associations with adverse health effects (*Guidance on PFAS Exposure, Testing, and Clinical Follow-Up*, 2022).

Placental trophoblasts are essential for many aspects of embryonic/fetal development, serving key roles in (1) physically anchoring the baby to the mother; (2) remodeling the uterine vascular architecture to enable increased blood flow to the placenta; (3) exchanging nutrients, wastes, and gases between the maternal and fetal units; (4) defending against pathogens; and (5) producing hormones (Fisher, 2015; Red-Horse *et al.*, 2004, 2005; Romero *et al.*, 2014). Placental trophoblasts may be particularly

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vulnerable to PFOA toxicity. In humans, prenatal exposures to PFOA are significantly associated with adverse outcomes commonly linked to impaired trophoblast function: increased risk of early miscarriage (Wikström *et al.*, 2021), preterm birth, preeclampsia, fetal growth restriction (Gao *et al.*, 2021), and low birth weight (Koustas *et al.*, 2014). Evidence in humans is largely supported by toxicological studies. For example, prenatal exposures to PFOA (oral; GD1.5–17.5) in CD-1 mice results in placental defects (Blake *et al.*, 2020). In human placental cell lines, PFOA compromises viability and migration (Szilagyi *et al.*, 2020b); while also disrupting vascular network formation (Poteser *et al.*, 2020). PFOA may perturb placentation by inducing apoptosis (Bangma *et al.*, 2020), altering endocrine function (Suh *et al.*, 2011), and impacting inflammatory signaling pathways (Poteser *et al.*, 2020; Szilagyi *et al.*, 2020b; Tsang *et al.*, 2013). Determining the effects of PFOA and other PFAS on trophoblast function and development is important to understand the mechanistic links between these compounds and pregnancy complications.

In this study, we utilized primary human cytotrophoblasts (CTBs) isolated from second trimester placentas, a period of rapid growth and hence high sensitivity, to characterize the impact of PFOA on their function and transcriptome. CTBs have been proposed as a model to study the effect of environmental chemicals on placental/trophoblast cell function (Robinson *et al.*, 2019). Using this model system, we show that PFOA is toxic to human CTBs, impairing cell viability and selectively impacts mRNA production. Our results suggest a multifaceted response to PFOA at the molecular level, suggesting links between human exposure to this chemical and adverse pregnancy outcomes.

Materials and methods

Human placenta collection

Informed consent was obtained from all donors and methods were approved by the UCSF Institutional Review Board. Placentas were collected following elective terminations (second trimester, gestational ages: 17.2–24 weeks) and immediately placed in cytowash medium, containing DME/H-21 (Gibco), 12.5% fetal bovine serum (Hyclone), 1% glutamine plus (Atlanta Biologicals), 1% penicillin/streptomycin (Invitrogen), and 0.1% gentamicin (Gibco). Tissue samples were placed on ice prior to dissection.

Human primary cytotrophoblast isolation and culture

As described previously (Chen *et al.*, 2021; Robinson *et al.*, 2017, 2019), CTBs were isolated from human placentas using enzymatic digestion and Percoll gradient density centrifugation. Single cells suspended in culture medium containing DME/H-21, 2% Nutridoma (Roche), 1% sodium pyruvate (Sigma), 1% Hepes buffer (Invitrogen), 1% glutamate plus (Atlanta Biologicals), and 1% penicillin/streptomycin (Invitrogen) were counted and transferred to tissue culture plates coated with Matrigel (BD Biosciences) at a density of 1 million (6-well plate) or 250,000 (24-well plate) cells per well. Cells were incubated at 37°C in 5% CO_2 and 95% air. We routinely estimated CTB purity via immunostaining with anti-cytokeratin (CK; anti-CK rat polyclonal; 1:100 [Damsky *et al.*, 1994]), a specific marker of these cells within chorionic villi. Only preparations ≥85% CTBs were used in these experiments (Supplementary Figure 1 and Tables 1 and 2).

Chemicals

Stocks were generated by dissolving PFOA (CAS 335-67-1, Sigma, 95% purity) in dimethyl sulfoxide (DMSO). In a previous study, we showed that the concentration of DMSO (0.1%) used in this study as a vehicle control does not significantly alter CTB function (ie, viability, invasion, migration) or change gene expression levels relative to test chemical exposures (Robinson *et al.*, 2019). For each experiment, PFOA was diluted 1:1000 (vol/vol) in prewarmed culture medium and this solution was used to achieve concentrations of 0.1, 1, 10, 25, 100, or 250 μM. As described previously (Robinson *et al.*, 2017), CTBs were allowed to attach to the Matrigel substrate (approximately 1–3 h) before they were exposed to control- or PFOA-containing medium. All assays (except qRT-PCR validation experiments) were conducted with both DMSO and medium only controls.

Cytotoxicity assays

CTBs were exposed to PFOA for 24 h. As described in Robinson *et al.* (2019), we assessed cell viability using the neutral red lysosomal uptake assay (Repetto *et al.*, 2008). In brief, conditioned medium was removed and wells were gently washed with PBS. Fresh medium containing 40 ng/ml of neutral red dye was added to each plate and incubated at 37°C. After 2 h, CTBs were washed with PBS and a solution of 50% ethanol, 1% acetic acid, and 49% deionized water was added to each well. The relative amount of neutral red dye released into the solution was evaluated by measuring absorbance at 540 nm using a Biotek Epoch spectrophotometer. In parallel, we evaluated cell death by quantifying relative lactate dehydrogenase (LDH) activity in the conditioned medium using the Cytotoxicity Detection Kit (Roche). Following the recommended manufacturer's protocol, we measured absorbance at 490 nm. For both assays, background absorbance readings were subtracted from mean absorbance values and values were expressed relative to the medium only control (=100%). Average relative percentages and corresponding standard error (SEM) were computed across the independent experiments ($n \geq 4$). We identified significant changes ($p < .05$) across all concentrations (ordinary 1-way ANOVA) or per concentration versus the vehicle control (Dunnett's multiple comparisons test).

RNA isolation

After a 24-h exposure, the medium was removed and lysis buffer was added to the culture dish. The lysate was collected and stored at −80°C. Later, RNA was isolated from CTB lysates using the Zymo RNA/DNA Micro Plus Extraction Kit (samples for RNA sequencing) or Qiagen RNeasy Micro Kit (samples for qRT-PCR analyses) following the manufacturer's protocol for adherent cells. We assessed RNA concentration and quality using a Nanodrop (Thermo Scientific) and an Agilent 2100 BioAnalyzer. Only RNA samples with 260/280 nm absorbance values between 1.9 and 2.1 and RIN > 9 were used in downstream analyses.

RNA sequencing

We exposed CTBs to concentrations 25 μM (subcytotoxic at 24 h) or 100 μM (approximately 15% loss in viability at 24 h) of PFOA, or 0.1% DMSO for 24 h, and collected cell lysates from biological replicates ($n = 3$; GW 21.3, 21.4, or 24). Additional control CTBs were exposed to medium only. Limited differences were observed between DMSO and medium-only controls (not shown). We performed mRNA sequencing (RNA-seq) of CTBs at QB3 Genomics (UC Berkeley, Berkeley, California, RRID: SCR_022170). cDNA and library construction were completed using 500 ng of mRNA. RNA-seq was performed on a HiSeq 4000 to obtain approximately 25 million reads per sample. FASTQ files were generated and aligned to the human genome using STAR. Aligned BAM files were processed using htseq to obtain read counts per million (CPM). CPM

were further preprocessed and normalized in R using the edgeR (v3.14.0) and limma (v3.28.14) packages. Genes with CPM values > 0.5 in at least 2 samples were kept for further data analysis (Supplementary Table 3). Normalization factors were calculated, raw counts were converted to $\log_2$ CPM, and the data were transformed using voom (Law *et al.*, 2014). We generated box plots depicting $\log_2$ CPM per sample to assess similarities in distributions across the sample set (ggplot2; Supplementary Figure 2) and performed unsupervised principal component analysis (PCA) to compare count distributions and overall expression patterns among samples (ggfortify).

Identification of differentially expressed genes and enriched pathways

Using log 2 voom-transformed counts, we determined differentially expressed (DE) genes using BRB-Array Tools (a program suite developed by Dr Richard Simon and the BRB-ArrayTools Development Team) and 2-factor generalized linear models for PFOA, controlling for batch (placenta). DE genes were defined by a cut-off of (uncorrected) $p < .05$ and an absolute fold change (FC) > 1.5 with either concentration as compared with the vehicle control (Supplementary Table 4). Genes ($n = 19$) that were not detected (CPM = 0) in 1 or more samples were removed to further reduce identification of false positives. Additional statistical measures used to control for false positives (eg, Bonferroni) were not applied due to a limited number of genes passing more-conservative thresholds (FDR < 5%), a common observation in toxicological studies using primary human cell models with limited sample numbers (Armstrong, 2014). We employed a secondary pairwise *t* test to determine the significance of genes at each concentration ($p < .05$). We conducted supervised PCA and hierarchical clustering using average linkage and Euclidean distance (TIGR MEV; Saeed *et al.*, 2006) of normalized counts and FC values. Functional enrichment analysis of Gene Ontology (GO) Biological Processes (Level 4) was evaluated via the Database for Annotation, Visualization and Integrated Discovery (Sherman *et al.*, 2022). We applied a cut-off criteria of $p < .005$ and specified the number of DE genes per term ($n > 5$) to identify overrepresented processes (Supplementary Table 5), which were classified by GO hierarchy (Gene Ontology Consortium, 2015). Raw and normalized data were deposited in the NCBI Gene Expression Omnibus.

qRT-PCR validation of the RNA-seq data

We confirmed targets (*CORTICOTROPIN RELEASING HORMONE* [*CRH*], *INTERFERON INDUCED PROTEIN WITH TETRATRICOPEPTIDE REPEATS 1* [*IFIT1*], *TNF SUPERFAMILY MEMBER 10* [*TNFSF10*]) in PFOA- (25 or 100 μM) or vehicle-exposed (0.1% DMSO) controls. We employed additional biological replicates ($n = 4$; GW = 18.2, 22.1, 23.2, or 24). We converted purified RNA to cDNA using iSCRIPT Universal TaqMan (Bio-Rad) and performed qRT-PCR using TaqMan Universal Master Mix II (no UNG, Life Technologies) and Taqman primers (Supplementary Table 6). All reactions were performed in triplicate for 40 cycles. We determined relative levels of expression between experimental and control samples by applying the ΔΔCT method, which is comprised of (1) normalizing to the mean of commonly used housekeeping genes (*GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* [*GAPDH*] and *ACTIN BETA* [*ACTB*]) (Arenas-Hernandez and Vega-Sanchez, 2013); and (2) adjusting to the vehicle control for each experiment. Expression levels of *GAPDH* and *ACTB* were not impacted by PFOA exposures in the RNA-seq data. To control for baseline differences in gene expression across biological replicates, we employed paired *t* tests to identify significant changes ($p < .05$) between each testing concentration versus the vehicle control (Microsoft Excel).

Transcription factor binding site enrichment analysis

We interrogated DE genes for enrichment of genomic binding sites associated with upstream regulatory transcription factors (TFs) using EnrichR (Han *et al.*, 2018) and the TRRUST Transcription Factor 2019 database. We explored overrepresentation of TF binding sites (TFBS) in (1) all DE genes; (2) upregulated genes; and (3) downregulated genes. We applied a cut-off of $p < .005$ and specified the number of DE genes (>3) to identify significantly enriched TFBS (Supplementary Table 7).

Estimating PPAR activity of PFOA and other PFAS

Using publicly available toxicological data available on the U.S. EPA dashboard (Lowe and Williams, 2021), we explored the activity of PFOA and other PFAS on PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR (PPAR) ALPHA (PPARA) and PPAR GAMMA (PPARG) activities as well as *PYRUVATE DEHYDROGENASE KINASE 4* (*PDK4*) expression. We identified "hits" (ie, compounds with significant activity) corresponding to AC50 values for cell-free (ATG_PPARa_TRANS_up; ATG_PPARg_TRANS_up) or cell-based (LTEA_HepaRG_PDK4_up) assays in the ToxCast database. We compared activity/AC50 values among PFAS and 2 known PPAR-agonists (pioglitazone hydrochloride and pirinixic acid [Peters *et al.*, 1997; Smith, 2001]).

Comparison of transcriptomic responses to PFOA versus pioglitazone

For these analyses, we used publicly available transcriptomic profiles of term CTBs exposed to pioglitazone (20 μM; $n = 6$) or a vehicle control ($n = 3$; GSE103993 [El Dairi *et al.*, 2018]) for 72 h. Raw data (probe intensities) generated using the Illumina microarray platform were normalized and expression differences between experimental and control CTBs were identified using 1-way ANOVA (NCI BRB Array Tools). Duplicates were removed from the analysis by using the most highly DE probe (based on *p* value). After processing, we coalesced this dataset with our PFOA analysis using the Official Gene Symbol. In total, we compared expression profiles of 10 749 genes. Using the same criteria (ANOVA, $p < .05$; absolute FC ratio > 1.5 for pioglitazone or PFOA vs their respective vehicle controls), we identified common DE genes and their FCs. "High" confidence targets based on the PPARgene database were denoted (Fang *et al.*, 2016).

Benchmark concentration response analysis

We quantified low-level perturbations in response to PFOA exposures using a benchmark concentration (BMC) modeling approach. First, we adjusted log 2-transformed counts by calculating FC values corresponding to the ratio of counts between each exposure group (ie, PFOA or DMSO) versus the concurrent medium-only control. Ninety-five percent of DE genes (419/443 genes) were examined in this context. Next, we applied BMDExpress 2 to calculate BMCs reflective of a 10% change using default settings (BMR = 1.349, 0.95 confidence level, constant variance, maximum iterations = 250). BMCs were calculated based on curves corresponding to standard statistical models: power, linear, or polynomial 2 models. The "best-fit model" was selected using the Akaike Information Criterion (AIC) to determine BMC and BMC lower-bound (BMCL; based on the lower 95th confidence limit) values. To reduce overfitting, we removed estimates where BMC/BMCL > 20 for the "best-fit model." In these cases, identified

by AIC, we estimated BMCs using linear (approximately 3.1% of genes) or poly 2 (approximately 2.6% of genes) models. All curves were analyzed to assure proper fitting. The latter analysis led to the removal of 5 genes with BMC estimates > 500 μM. In total, BMC estimates were calculated for 414 DE genes and categorized by enriched GO biological processes (Supplementary Table 5).

Comparing our results and related studies

We compared estimated PFOA concentrations that induce functional and molecular changes in CTBs, with other *in vitro* model that have been used to assess human placental toxicity. We applied a Microsoft Excel-based tool to search for keywords in PubMed (AbstractSifter [Baker *et al.*, 2017]; search terms: "perfluorooctanoic acid" and "placenta") to identify *in vitro* studies investigating PFOA toxicity of placental cell lines or primary CTBs.

Each abstract was reviewed to assure relevancy. In total, we identified 9 studies (Supplementary Table 10). PFOA effects were summarized and categorized by molecular, cellular/functional, or morphological endpoints. We further stratified effects using a qualitative scale and reported as follows: (1) significant; (2) trending, approaching significance; or (3) nonsignificant.

Results

PFOA induces cytotoxicity in CTBs

PFOA significantly reduced cell viability and increased LDH activity in a concentration-dependent manner ($p < .05$, ANOVA; Figure 1). In pairwise comparisons between each concentration and the vehicle control, we observed significant effects with 250 μM ($p < .05$; Dunnett's), corresponding to a 45.4% reduction in viability and a 37.1% increase in LDH activity. Similar trends were observed with 100 μM, however, these changes were more modest (15.1% loss in cell viability; 10.4% increase in LDH activity; $p > .05$). Therefore, we demonstrated that PFOA induces cytotoxicity in a concentration-dependent manner in second trimester primary human CTBs.

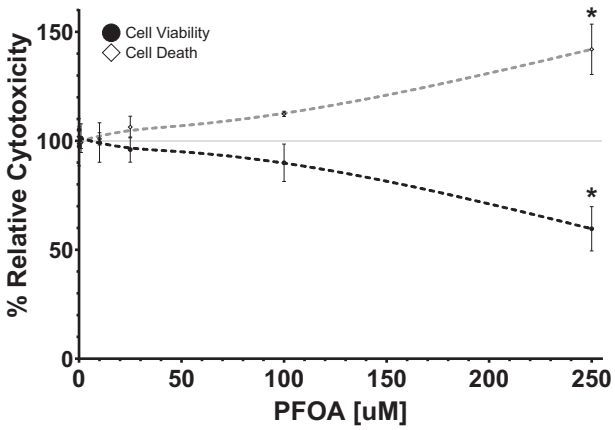


Figure 1. PFOA exposures induce cytotoxicity in primary CTBs. Relative cell viability (neutral red uptake) and death (lactate dehydrogenase activity) in PFOA or vehicle (0.1% DMSO) exposed CTBs at 24 h. Values were adjusted to the medium only control (=100%) for each experiment–unexposed CTBs from different placentas ($n \geq 3$ second trimester, matched experimental and control cultures). Dashed lines represent spline curves. Asterisks (*) note significant differences between specific concentrations of PFOA and the vehicle control ($p < .05$; Dunnett's multiple comparisons test).

PFOA alters the CTB transcriptome in a concentration-dependent manner

PCA analysis of all genes revealed significant separation in samples by placental origin, but not by PFOA exposure (25 or 100 μM) (Figure 2A). We implemented multiple linear regression to identify DE genes due to PFOA exposure while accounting for placental origin. In total, 443 genes were identified to be DE with PFOA (ANOVA, $p < .05$; absolute FC > |1.5| with either concentration of PFOA vs vehicle control; Supplementary Table 4). Using this subset of DE genes, we observed significant separation of samples by placental origin and by the 3 tested concentrations via PCA (Figure 2B). In general, DE genes trended in a monotonic fashion; larger changes were observed with 100 μM versus 25 μM (Figure 2C). For genes within this subset, we performed pairwise statistical comparisons between each concentration of PFOA and the vehicle control and found approximately 2X (225 vs 118 genes) as many genes to be disrupted with 100 μM versus 25 μM ($p < .05$; Figure 2D). A higher proportion of up- versus downregulated genes was observed with 25 μM (97/21), whereas with 100 μM, we observed a similar number of up- versus downregulated genes (108/117). Our results indicated that PFOA, in a concentration-dependent manner, consistently disrupted gene expression in primary human CTBs from different individuals.

We examined the 25 most highly up- (Figure 3A) or downregulated (Figure 3B) genes as determined by FC with either 25 or 100 μM exposures. The most upregulated genes included molecules involved in: glucose/lipid metabolism (*PDK4*, *DEHYDROGENASE/REDUCTASE 9* [*DHRS9*] and *12* [*DHRS12*] and *ELOVL FATTY ACID ELONGASE 4* [*ELOVL4*]); hormone signaling (*CRH*, *ANGIOPOIETIN LIKE 4* [*ANGPTL4*]); transport ([*PERILIPIN 2* [*PLIN2*], *SOLUTE CARRIER FAMILY 11 MEMBER 1* [*SLC11A1*] and *SOLUTE CARRIER FAMILY 6 MEMBER 17* [*SLC6A17*]); and trophoblast fusion (*ENDOGENOUS RETROVIRUS GROUP FRD MEMBER 1, ENVELOPE* [*ERVFRD-1*]). The most downregulated genes included molecules linked with: trophoblast function (eg, *TUMOR PROTEIN P63* [*TP63*] and *KERATIN 6A* [*KRT6A*]); innate immune response (eg, *MX DYNAMIN LIKE GTPASE 1 AND 2* [*MX1*, *MX2*], *IFIT1-3*, *TOLL LIKE RECEPTOR 3* [*TLR3*], *2′-5′-OLIGOADENYLATE SYNTHETASE 3* [*OAS3*], *GUANYLATE BINDING PROTEIN 4* [*GBP4*], and *DEXD/H-BOX HELICASE 60* [*DDX60*]); and apoptosis (eg, *TNFSF10* and *XIAP ASSOCIATED FACTOR 1* [*XAF1*]). Using CTBs from additional human placentas ($n = 4$), we validated differential expression of 3 of these highly modulated targets (*CRH*, *IFIT1*, *TNFSF10*) via qRT-PCR (Figure 3C). In a concentration-dependent manner, PFOA altered expression of all 3 genes: significant effects were observed with 25 μM for *TNFSF10* and 100 μM for all 3 genes (paired *t* test, $p < .05$). Patterns of altered expression were highly correlated between the RNA-seq and qRTPCR data ($R^2 = 0.98$, Supplementary Figure 3). Our analysis highlighted the expression of specific molecules perturbed by PFOA exposure of human CTBs.

Enrichment analysis of genes altered by PFOA

We performed GO analysis to identify enriched biological processes associated with all PFOA DE genes as well as the up- and downregulated subsets. In total, we observed 30 GO terms were overrepresented ($p < .005$; number genes changed associated with term > 5; Supplementary Table 5). Significantly enriched terms were related to inflammation, innate immunity, lipid metabolism, signaling, and programmed cell death (select examples shown in Figure 4A). The most significantly enriched terms were associated with inflammation and immunity pathways (response

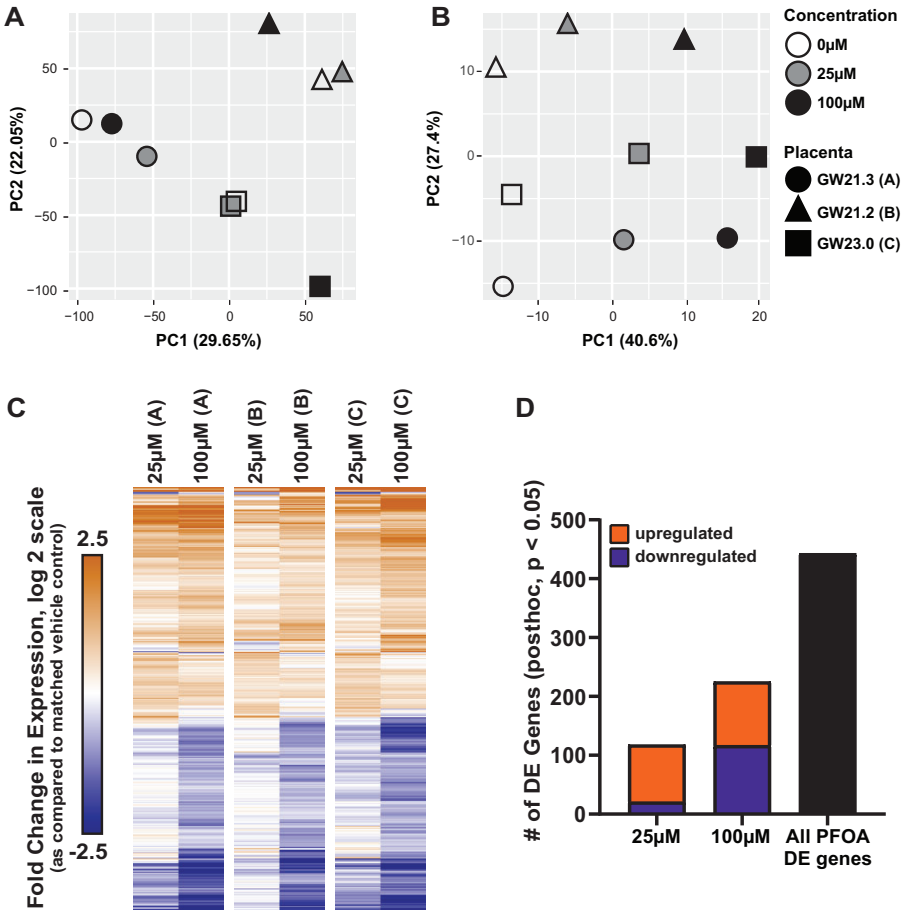


Figure 2. PFOA exposures alter the CTB transcriptome. Principal component analysis (PCA) of CTB samples for all genes (A) and those differentially expressed (DE) with PFOA exposure (B). Hierarchical clustering of genes identified as DE following 2 concentrations of PFOA exposure ($n = 443$ unique genes; ANOVA, $p < .05$, absolute average fold change [FC] > 1.5) (C). Color intensities represent relative expression of each gene as compared with the matched vehicle control (0.1% DMSO) in each experiment ($n = 3$). Number of genes whose expression was altered by exposure to each concentration of PFOA (vs vehicle control; paired *t* test, $p < .05$; D). GW = gestational week.

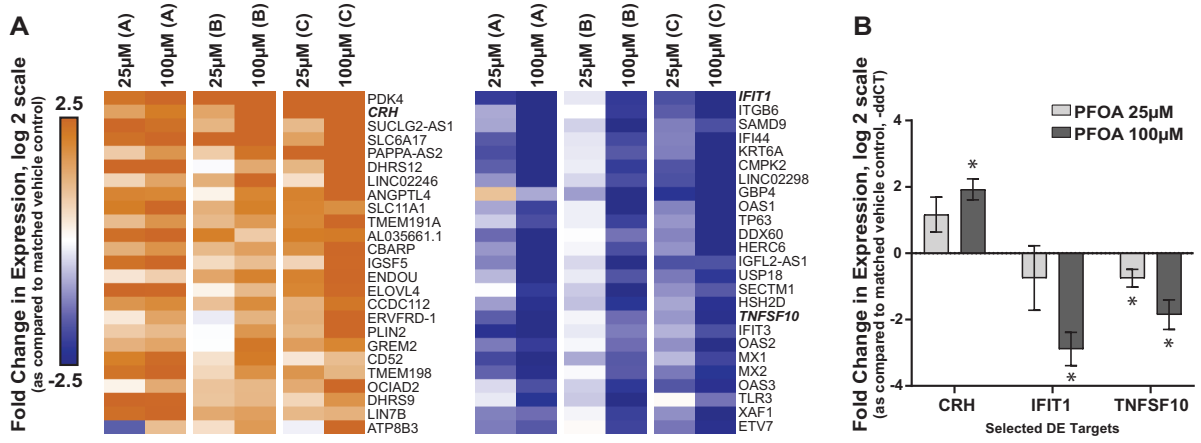


Figure 3. PFOA exposures alter CTB expression of many biologically important targets. The 25 most highly up- or downregulated differentially expressed (DE) genes based on fold change differences in expression between PFOA and vehicle control (A). The expression of italicized genes was independently analyzed using additional biological samples (see panel B). qRT-PCR validation of PFOA-induced perturbations in expression of *CORTICOTROPIN RELEASING HORMONE* (*CRH*), *INTERFERON INDUCED PROTEIN WITH TETRATRICOPEPTIDE REPEATS 1* (*IFIT1*), or *TNF SUPERFAMILY MEMBER 10* (*TNFSF10*) ($n \geq 3$ biological replicates; B). Asterisks represent significant differences between PFOA exposure versus vehicle control (paired *t* test, $p < .05$).

to type I interferon [$p = 4.8E\text{-}10$]; response to cytokine [$p = 3.9E\text{-}07$]; response to virus [$p = 4.09E\text{-}07$]; negative regulation of viral process [$p = 1.9E\text{-}06$]; innate immune response [$p = 2.8E\text{-}06$]; and defense response to virus [$p = 4.6E\text{-}06$]). These pathways tended to be more highly associated with DE genes identified as downregulated by PFOA exposure. Other enriched terms such as cell surface receptor signaling pathway and programmed cell death also included more highly down- versus upregulated genes. In

contrast, categories such as cellular lipid metabolic process, lipid modification, and fatty acid metabolic process were enriched in only the upregulated subset of DE genes and predominately included genes upregulated by PFOA exposure.

We further explored DE gene subsets associated with cellular lipid metabolism or innate immune response due to their previous hypothesized roles in PFOA toxicity and their association with divergent trends in up- versus downregulated genes. PFOA altered 31 (↑22, ↓9) genes associated with cellular lipid metabolism (Figure 4B). This upregulated subset of molecules included those important for acetyl coenzyme a (acetyl-CoA) metabolism (*ACYL-COA DEHYDROGENASE VERY LONG CHAIN* [*ACADVL*], *ACETYL-COA ACYLTRANSFERASE 2* [*ACAA2*], *ACYL-COA SYNTHETASE LONG CHAIN FAMILY MEMBER 4* [*ACSL4*], *CARNITINE PALMITOYLTRANSFERASE 1A* [*CPT1A*], *ELECTRON TRANSFER FLAVOPROTEIN-DEHYDROGENASE* [*ETFDH*] and its subunits, including *ALPHA* [*ETFA*] and *BETA* [*ETFB*]; glucose metabolizing enzymes [*PDK2* and *PDK4*]). Other molecules with altered upregulated mRNA expression included *CELLULAR RETINOIC ACID BINDING PROTEIN 2* (*CRABP2*), *PROTEIN TYROSINE KINASE 2 BETA* (*PTK2B*), *SYNDECAN 1* (*SDC1*), *DHRS9* and *CRH*. Downregulated targets associated with PFOA exposure included molecules involved in phospholipid metabolism: *LIPASE C, HEPATIC TYPE* (*LIPC*), *PATATIN LIKE PHOSPHOLIPASE DOMAIN CONTAINING 3* (*PNPLA3*), *PHOSPHATIDYLINOSITOL-5-PHOSPHATE 4-KINASE TYPE 2 BETA* (*PIP4K2B*), *PHOSPHOLIPID PHOSPHATASE 3* (*PLPP3*), and *PHOSPHOLIPID SCRAMBLASE 1* (*PLSCR1*). Thirty-nine DE genes (↑4; ↓35) were linked with the GO term, innate immune response (Figure 4C). Upregulated genes included *ANKYRIN REPEAT AND KH DOMAIN CONTAINING 1* (*ANKHD1*), *PTK2B*, and *SLC11A1*. Downregulated genes consisted of pro-inflammatory chemokines (*CCL2*, *CCL5*); interferon-induced proteins (eg, *IFIT-1*,-3, and -5, *INTERFERON INDUCED WITH HELICASE C DOMAIN 1* [*IFIH1*], *INTERFERON INDUCED PROTEIN 35* [IFI35], *INTERFERON ALPHA INDUCIBLE PROTEIN 6* [*IFI6*] and *ADENOSINE DEAMINASE* [*ADAR*]); molecules critical to antiviral response (eg, *OAS1-3*, *DDX60*, *MX1* and *MX2*, *SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 1* [*STAT1*], and *TLR3*); and complement factors (eg, *COMPLEMENT C1s* [*C1S*], *COMPLEMENT C2* [*C2*], and *COMPLEMENT FACTOR B* [*CFB*]). Thus, our results highlighted numerous molecules linked with lipid metabolism and inflammation/innate immunity to be altered by PFOA exposure in CTBs.

Transcription factor binding analysis of genes altered by PFOA

We assessed all DE genes and up- and downregulated subsets for enrichment of TFBS to determine potential upstream regulatory targets of PFOA exposure. The most highly overrepresented TFBSs included INTERFERON REGULATORY FACTOR 1 and 3 (IRF1 and IRF3), REL-ASSOCIATED PROTEIN (RELA), NUCLEAR FACTOR KAPPA B (NFKB), STAT-1 and -3, HISTONE DEACETYLASE 1 and 3 (HDAC1 and HDAC3), and SPI-1 PROTO-ONCOGENE (SPI1; Figure 5A; Supplementary Table 6). These TFBSs were predominately associated with downregulated DE genes in response to PFOA exposure. In contrast, peroxisome proliferator-activated receptors, PPARA and PPARG, were exclusively linked with DE genes upregulated by PFOA exposure. STAT1 was also found to be significantly downregulated on the mRNA level in our analysis. Other TFs displayed nonsignificant changes on the transcriptomic level based on our analysis criteria.

The PPAR receptor family plays key physiological roles, including lipid homeostasis and inflammation (Abbott, 2009; Wahli and Michalik, 2012), and also are involved in human trophoblast function (Barak *et al.*, 1999, 2008; Fournier *et al.*, 2011). As the PPAR receptor family has been implicated in PFOA toxicity (Abbott *et al.*, 2012), we further explored the relationship between PPAR activity and PFOA exposures using publicly available datasets accessed through the U.S. EPA dashboard (Lowe and Williams, 2021). Specifically, we used those related to PPARA or PPARG activity or induced PDK4 expression, a known transcriptional target of PPAR. For these assays, we compiled AC50 values for 2 known selective PPAR agonists (pioglitazone hydrochloride and pirinixic acid), PFOA and 7 other PFAS shown to be toxic in a human trophoblast cell line (Blake *et al.*, 2022). This *in silico* analysis predicted that PFOA induced PPARA and PPARG transcriptional activity, as well as *PDK4* expression, at concentrations (AC50) similar to other PFAS and within 1–2 orders of magnitude of the PPAR agonists (Figure 5B).

To identify CTB PFOA targeted genes with PPAR activity, we compared transcriptomic responses to PFOA (25 and 100 μM) versus pioglitazone by leveraging a publicly available transcriptomic dataset of term CTBs exposed to pioglitazone (20 μM) (El Dairi *et al.*, 2018). Although the invasiveness and motility of extravillous CTBs in term placentae are significantly reduced compared with earlier gestational ages, it was anticipated that a subset of the target genes in these cell populations would be similarly susceptible to PFOA and pioglitazone exposure (Knöfler *et al.*, 2019). We identified 10 749 genes as shared between the 2 datasets. Of these, 356 were DE with PFOA exposure and 245 were DE with pioglitazone exposure (ANOVA, $p < .05$; absolute FC ratio $>|1.5|$). In total, 21 genes were DE in both datasets and had similar expression patterns (shaded circles; Supplementary Table 8).

Responses to both concentrations of PFOA–25μM ($R^2 = 0.4316$; $p = .0012$; Figure 5C) or 100 μM ($R^2 = 0.4968$; $p = .0040$; Figure 5D) were significantly correlated with those to pioglitazone exposure. Common DE genes previously recognized as PPAR targets (Fang *et al.*, 2016) included: *PLIN2*, *PDK4*, *ANGPTL4*, *ACADVL*, *MONOCYTE TO MACROPHAGE DIFFERENTIATION ASSOCIATED* (*MMD*). Other genes commonly altered by PFOA and pioglitazone exposures were: (upregulated) *CD52 MOLECULE* (*CD52*), *CRH*, *DHRS9*, *ELOVL4*, *FOUR AND A HALF LIM DOMAINS 1* (*FHL1*), *GROWTH HORMONE 2* (*GH2*), *LIF RECEPTOR SUBUNIT ALPHA* (*LIFR*), *LYMPHOCYTE ANTIGEN 6 FAMILY MEMBER E* (*LY6E*), *OLFACTOMEDIN LIKE 2B* (*OLFML2B*), *SLC11A1*, *STATHMIN 1* (*STMN1*), and *TCL1 FAMILY AKT COACTIVATOR B* (*TCL1B*) and (downregulated) *CCL5*, *LAMININ SUBUNIT GAMMA 2* (*LAMC2*) and *KRT6A*. Of note, based on FC, *FATTY ACID-BINDING PROTEIN 4* (*FABP4*) was the most upregulated gene in both datasets. However, significance was achieved only with pioglitazone ($p = .0001$) and not PFOA ($p = .06$). Our results suggest specific TFs such as IRFs and PPARs may mediate PFOA-induced CTB toxicity.

Benchmark concentration analysis revealed sensitive PFOA targets in placental CTBs

We determined best-fit models for each DE gene and calculated relative BMCs associated with a 10% shift in response to identify sensitive transcriptional changes induced by PFOA exposure. For the majority of genes, the linear model ($n = 266$ genes; 64.3%) had the best-fit followed by Polynomial 2 ($n = 141$; 34.1%) and Power ($n = 7$; 1.7%) models (Supplementary Table 9). BMCs ranged over approximately 2 orders of magnitude (3.8 μM—437.1 μM; Figure 6A). The median BMC and BMCL were 39.5 μM and 25.5 μM,

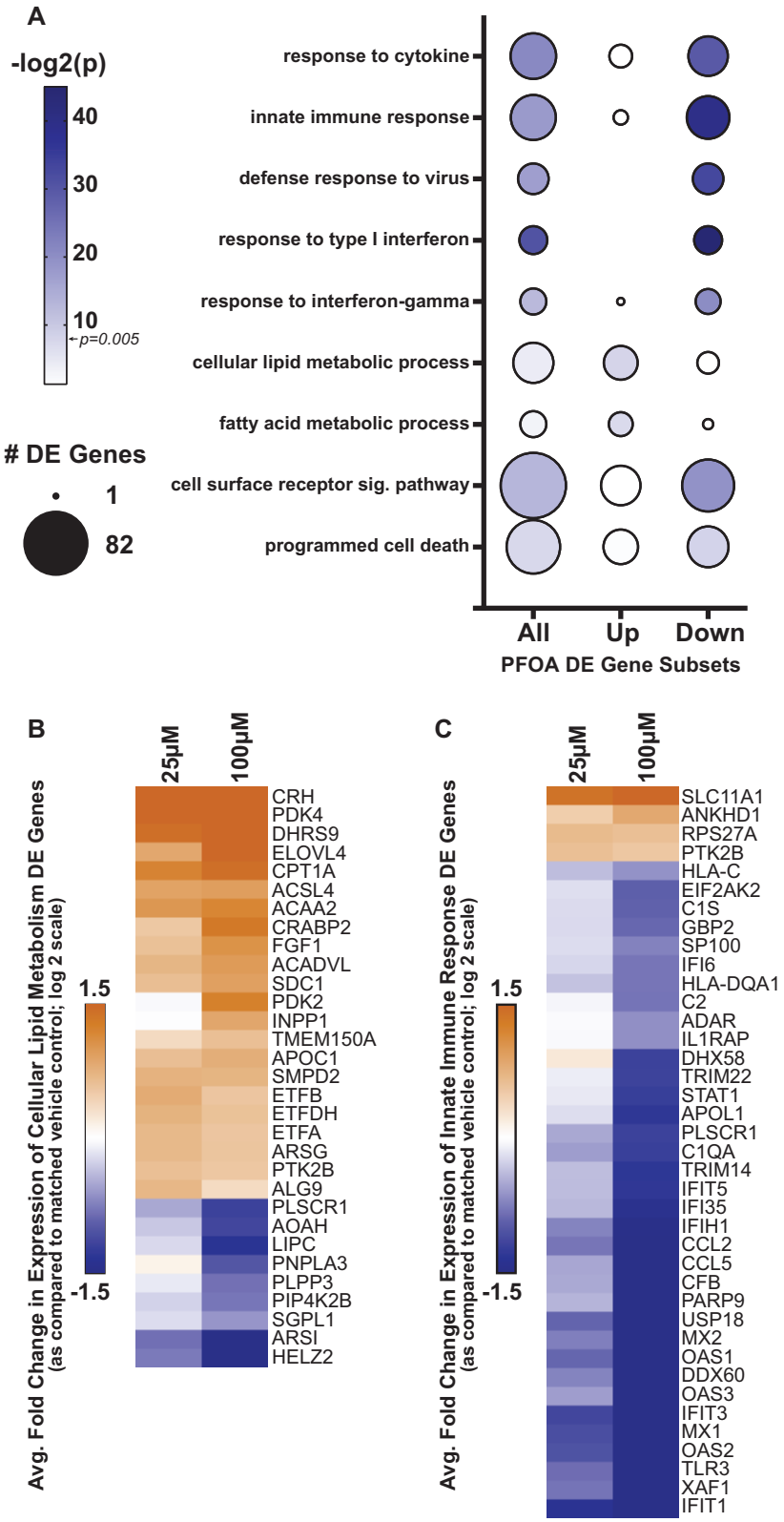
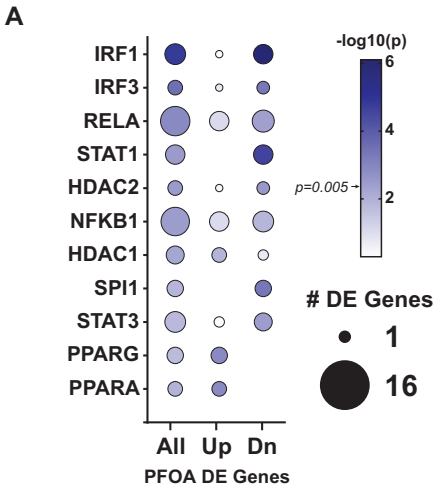


Figure 4. PFOA exposure of CTBs impacted specific biological processes. Enriched gene ontology (GO) biological processes for all DE genes and up- and downregulated subsets ($p < .005$; number of DE genes linked to term ≥ 6; A). Shading and size of bubbles correspond to (1) significance of enrichment ($-\log p$) and (2) number of DE genes associated with each GO term. Average relative expression of DE genes linked with cellular lipid metabolism (B) and innate immune response (C).

respectively. The 5% most sensitive transcripts to PFOA exposure were predicted to be perturbed at concentrations $\leq$ 8.4 μM (Figure 6A, inset) and included molecules such as *G PROTEIN SUBUNIT ALPHA O1* (*GNAO*; BMC = 3.8 μM); *PEPTIDYL-TRNA HYDROLASE DOMAIN CONTAINING 1* (*PTRHD1*; 4.2 μM); *ANGPTL4* (4.9 μM); *KRT6A* (5.1 μM); *ZINC FINGER PROTEIN 346* (*ZNF346*;



B

		USEPA TOXCAST		
	Class	PPARA (AC50)	PPARG (AC50)	PDK4 (AC50)
Pioglitazone hydrochloride	Agonist	5.0	0.1	11.4
Pirinixic acid	Agonist	5.6	18.8	22.4
Perfluorononanoic acid	PFNA; C9	14.3	43.4	33.0
Perfluorooctanoic acid	PFOA; C8	21.8	49.6	24.8
Perfluoroundecanoic acid	PFDA; C11	26.8	40.7	Inactive
Perfluorohexanoic acid	PFHxA; C6	36.2	Inactive	20.9
Perfluorooctanesulfonic acid	PFHpA; C8	58.9	26.7	24.2
Perfluoroheptanoic acid	PFAS; C7	15.9	105.7	48.3
Perfluorodecanoic acid	PFUnDA; C10	31.3	57.2	24.6
Perfluorooctanesulfonamide	PFOSA; C8	83.1	84.0	Inactive

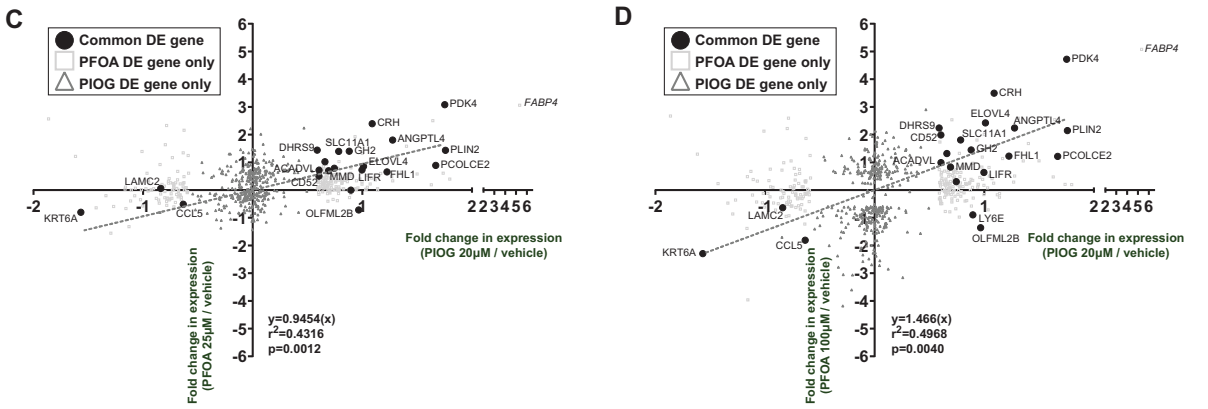


Figure 5. Predicted relationship between PFOA exposures and CTB transcription factor activity. Enrichment of transcription factor binding sites associated with PFOA responses for all differentially expressed (DE) genes and up- and downregulated subsets (A). Shading and size of bubbles correspond to (1) significance of enrichment (−log *p*) and (2) number of DE genes associated with each transcription factor binding site (TFBS). Bioactivities of PPAR agonists or PFAS read out in terms of PPARA/PPARG activity or PDK4 expression (Lowe and Williams, 2021). The PFAS shown were toxic in a human trophoblast cell line (Blake *et al.*, 2022). Their carbon length is denoted (B). Comparisons of CTB transcriptomic responses to pioglitazone (PIOG) versus PFOA 25 μM (C) or PFOA 100 μM (D). Black circles represent DE genes in both studies.

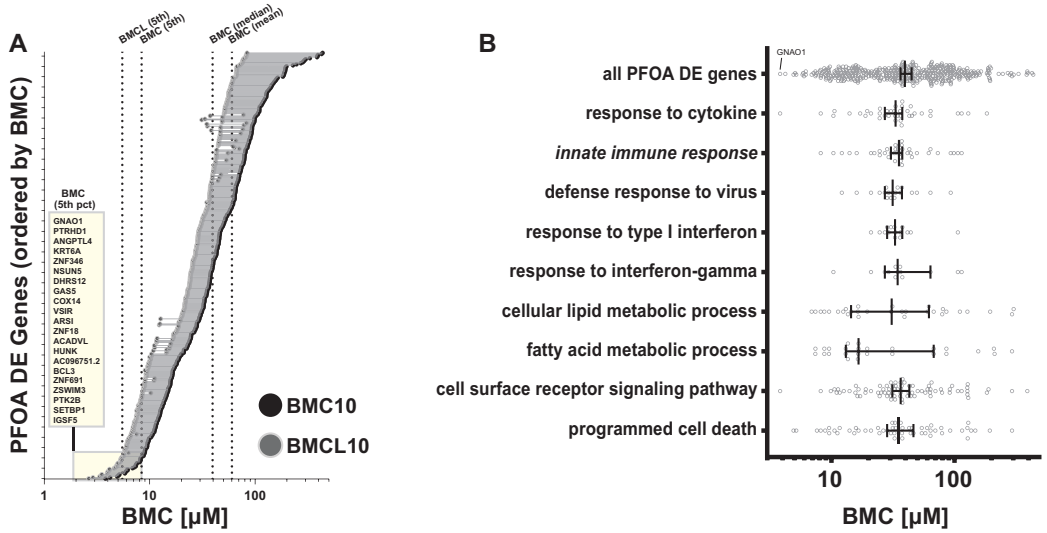


Figure 6. CTB transcripts and pathways predicted to be sensitive to PFOA exposure. Distribution of estimated benchmark concentrations (BMCs) and associated BMCLs (lower bound estimate) for DE genes at 24 h. In total, BMCs were estimated for 414 DE genes (Figure 2). Dashed lines represent (1) BMCL, fifth percentile; (2) BMC, fifth percentile; (3) BMC, median; or (4) BMC, mean. Shaded box displays gene names of transcripts whose expression is predicted to be most sensitive to PFOA exposures (ie, lowest fifth percentile) (A). Genes whose BMC values are shown in panel (A) were mapped to enriched GO Biological Processes. Italicized GO terms are associated with DE genes that were predicted to be highly sensitive to PFOA exposures versus all DE genes ($p < .05$; *t* test; B).

5.2 μM); *NOP2/SUN RNA METHYLTRANSFERASE 5* (*NSUN5*; 5.8 μM); and *DHRS12* (5.8 μM).

Finally, we mapped enriched GO biological processes shown in Figure 4A and Supplementary Table 5 to BMCs of DE genes. On average, these BMCs, which linked to GO terms associated with immunological responses or lipid biology, were lower than the average of all BMCs attributed to genes whose expression was altered by PFOA exposure. Specific pathways predicted to be significantly more responsive to PFOA versus the complete DE gene set ($p < .05$; Figure 6B and Supplementary Table 5) included: innate immune response (median BMC = 35.4 μM), response to virus (30.4 μM), response to other organism (32.5 μM), defense response to other organism (31.4 μM), and lipid oxidation (13.3 μM). These results suggested specific molecules and their related pathways were differentially responsive to PFOA exposure in CTBs.

Discussion

Using primary human CTBs isolated from second trimester placentas, we showed that PFOA alters CTB viability/cell death and global gene expression. We also provide evidence that specific pathways, which are critical for normal placental function and disease, are perturbed by PFOA exposure. Using complementary cell viability and cell death assays, we showed that PFOA induced cytotoxicity in a concentration-dependent manner with significant changes occurring at micromolar concentrations (≥250 μM; Figure 1). This finding is in line with previous estimates made in human placental cell lines using similar exposure paradigms (Blake *et al.*, 2022; Du *et al.*, 2022; Gorrochategui *et al.*, 2014; Szilagyi *et al.*, 2020b). PFOA exposure also alters other important trophoblast functions including proliferation (Blake *et al.*, 2022), migration (Blake *et al.*, 2022; Szilagyi *et al.*, 2020b), and attachment (Tsang *et al.*, 2013). These reports are supported by studies in mice linking *in utero* PFOA exposure to impaired placental function and/or defects (Blake *et al.*, 2020; Jiang *et al.*, 2020). As a whole, these investigations point to PFOA as a potential disruptor of trophoblast function.

We profiled the effects of CTB exposure to PFOA on the transcriptomic level to determine molecular perturbations that could alter trophoblast function and contribute to pathological changes. Gene expression patterns were perturbed in a concentration-dependent manner and were consistent across biological replicates. We identified hundreds of genes as altered (Figure 2), including those involved in pathways critical for placental development. Our study represents the first investigation to profile the transcriptome of primary human CTBs in the context of PFOA exposure, expanding upon previous more targeted investigations in human or monkey placental cell lines (Supplementary Table 10).

Lipids and sterols are critical for proper fetal development and impaired balance of these components during pregnancy is linked to preeclampsia (Khaire *et al.*, 2021), fetal growth restriction (Alahakoon *et al.*, 2020), and preterm labor (Chavan-Gautam *et al.*, 2018) as well as neurodevelopmental disorders at later stages of life (Tarui *et al.*, 2022). In primary CTBs, PFOA altered genes with varied functions in lipid metabolism (Figs. 4A and 4B). Our analysis revealed several interesting upregulated targets involved in lipid oxidation, including enzymes involved in the promotion of pools of acetyl-CoA in the mitochondria. Specifically, cytosolic CPT1A catalyzes the transformation of acyl-CoA (derived from free fatty acids) to acylcarnitine, which facilitates transportation of acetyl-CoA to the mitochondria. ACAA2 catalyzes the last step in the fatty acid oxidation spiral, supporting the conversion of acylcarnitine to acetyl-CoA and NADH (Pietrocola *et al.*, 2015). ACADVL is essential for the breakdown of long-chain fatty acids that feed into this pathway (Chen *et al.*, 2020). Genes linked with GO terms such as cellular lipid metabolic process, fatty acid metabolic process, and lipid oxidation were among the most sensitive pathways affected by PFOA exposure (Figure 6 and Supplementary Table 5). These results suggested that PFOA may alter fatty acid metabolic pathways, placental lipidogenesis, and bioavailability of metabolic components to the embryo/fetus.

CRH, a protein-encoding gene associated with cellular lipid metabolism and induced by PFOA (Figs. 3A and 3C), is a key placental hormone that is implicated in the initiation of human labor (Wadhwa *et al.*, 1998). Interestingly, CRH is elevated in preterm birth cases versus controls (Perng *et al.*, 2020). Although the link between PFAS and preterm birth remains uncertain, in a recent study of women with elevated stress, higher PFAS levels were associated with elevated serum levels of CRH at mid-gestation (Eick *et al.*, 2022). In addition, PFOA increased expression of dehydrogenases important for hormone regulation. DHRS9 is involved in the conversion of 3-alpha-tetrahydroprogesterone to dihydroxyprogesterone and 3-alpha-androstanediol to dihydroxyprogesterone, which could decrease progesterone bioavailability, a risk factor for preterm birth. Although the role of DHRS12 in progesterone metabolism is less well-understood, this enzyme has been implicated in typical features that are also key for terminal extravillous trophoblast cells, such as proliferation, migration, and invasion (Xu *et al.*, 2020). Thus, we provide evidence that PFOA perturbed expression of several targets that may contribute to altered placental function and development.

Although the mechanisms remain undefined, PFOA–in a wide range of experimental models (Szilagyi *et al.*, 2020a; Yamamoto *et al.*, 2015)–disrupts the activities of PPAR, nuclear receptors with major regulatory roles in energy homeostasis and metabolic function. Three PPAR isotypes have been identified in humans (PPARA, -B, and -G), and all 3 receptors are expressed in human trophoblasts (Wang *et al.*, 2002). In particular, PPARG is important for placental function as it is required for lipid accumulation (Barak *et al.*, 1999), trophoblast epithelial differentiation (Fournier *et al.*, 2008), and extravillous trophoblast migration (Fournier *et al.*, 2002; Tarrade *et al.*, 2001). Our study provides new evidence that PFOA may alter PPAR activity in human CTBs. We observed an enrichment of PFOA-upregulated DE genes with PPARA and PPARG binding sites (Figure 5A). Genes known to be regulated PPAR–such as PDK4, PLIN2 and ANGPTL4, and ELOVL4–were among the most highly upregulated targets in our dataset (Figure 3A). We also discovered a high correlation of transcriptomic responses of CTBs induced by PFOA and pioglitazone, a known PPARG agonist (Figs. 5C and 5D). Our results are evidence of a role for PPAR in PFOA-induced CTB toxicity. These findings are of high relevance due to the potential synergistic activity of PFOA with other environmental chemicals (eg, PFAS [Figure 5B], triphenyl phosphate [Pillai *et al.*, 2014], phthalates [Shoaito *et al.*, 2019]) in promoting PPAR activity and the known role of PPAR in trophoblast function.

Inflammation and innate immunity pathways are critical for placentation, trophoblast invasion, and pathogen susceptibility (Robbins and Bakardjiev, 2012). Previous studies in nonplacental models suggest that PFOA may have broad effects on the immune system, modulating programming and levels of inflammatory mediators. On a transcriptomic level, PFOA altered CTB expression of molecules that are integral components of these

pathways (Figure 4A and Supplementary Table 5). GO terms related to innate immunity response/inflammation were the most significantly overrepresented in our analysis. In general, PFOA exposure downregulated expression of genes associated with these pathways (Figure 4C). Targets in this category included chemokines (CCL2, CCL5) involved in proinflammatory activities (Soria and Ben-Baruch, 2008) as well as molecules implicated in vulnerability and response to pathogens such as antiviral translational regulators (IFIT1 [Ambühl *et al.*, 2017], IFIT3, IFIT5); dynamin like GTPases (MX1, MX2); 2'-5'-oligoadenylate synthetases (OAS1, OAS2, OAS3); and a Toll-like receptor (TLR3) that plays fundamental roles in pathogen recognition and innate immune activation (Koga and Mor, 2008). Our results expand upon a recent investigation demonstrating that PFOA (approximately 24 μM) reduces CCL2 protein expression and inhibits TLR3 mRNA production in a human trophoblast cell line (Szilagyi *et al.*, 2020b). Although PFOA has been shown to act as an immunosuppressant in humans and several experimental models(Chang *et al.*, 2016), its effect on immune pathways in the human placenta remain undefined. Future experiments should interrogate the relationship between PFOA (and other PFAS) and modulation of innate immune signaling pathways at the maternal-fetal interface. Many pathogens are capable of crossing the placental barrier and/or induce placental inflammation (eg, syphilis, listeria, cytomegalovirus, Zika virus), leading to serious pregnancy complications including fetal death, preterm labor, and severe neonatal diseases (Robbins and Bakardjiev, 2012). Our study identified specific targets and pathways important for pathogen susceptibility that may be modulated by PFOA. Future studies using expanded numbers of biological samples could provide better insight into effective concentrations that induce molecular and cellular perturbations in the placenta as well as factors (eg, sex, gestational age, genetic variability) that alter susceptibility.

Human relevance of in vitro *exposure levels using placental models*

We compiled peer-reviewed studies examining the effects of PFOA exposures in human placental, cell culture models. Information regarding PFOA-induced bioactivities on molecular, cellular/functional, or morphological levels were classified by study, model, and exposure level (Supplementary Table 10). Primary human trophoblasts present numerous advantages to traditional trophoblast cell lines, as considerable genomic differences exist between the different experimental models (Bilban *et al.*, 2010) and even between trophoblast cell lines (Burleigh *et al.*, 2007). The extent of differences between the methylation profiles of primary cells and trophoblast cell lines is likely sufficient to impart notable functional differences (Novakovic *et al.*, 2011), and thus interpretation of data generated from the latter model system should be mindful of those caveats especially in the context of environmental chemical exposures. Nevertheless, trophoblast cell lines represent accessible surrogate models and have provided important scientific insight into trophoblast function. In total, we identified 9 studies that fit our search criteria. In general, concentrations that induced effects in CTBs at 24 h in this study were in line with previous reports that identified broad effects on expression of placental hormones and inflammatory mediators, migration and cell viability. Short-term exposures that produced adverse effects in primary cells or cell lines overlapped with physiologically relevant levels of PFOA-occupational exposures measured in blood. However, these levels were 2–3 orders of magnitude higher than measurements of blood levels in U.S. women (95th percentile) (Woodruff *et al.*, 2011). These comparisons suggest concentrations that induce perturbations in CTBs and other placental cell models are relevant for human exposures, but generally exceed typical chronic blood levels of PFOA in U.S. women. However, these extrapolations should be made with caution. Primary cells are derived from genetically unique individuals and differ in gestational age and sex, which are hypothesized to be important cofactors in PFAS toxicity. BMC estimates were based on 3 levels (eg, vehicle control and 2 concentrations of PFOA), thus, limiting prediction of the biological consequences of exposures in the lower-concentration range. Effects due to acute exposures may not reflect alterations due to lower level, chronic exposures across gestation. Furthermore, many PFAS, including those detected in fetal tissues, are hypothesized to cause toxicity via similar mechanisms (eg, PPAR activation; Szilagyi *et al.*, 2020a). Thus, they could synergize in perturbing placental function.

Growing evidence suggests PFOA causes placental toxicity and contributes to adverse developmental outcomes associated with trophoblast dysfunction. Using primary human CTBs isolated from second trimester placentas, we demonstrated that PFOA alters CTB viability/cell death and, at subcytotoxic concentrations, global gene expression. We identified molecules and pathways disrupted by PFOA that are critical for placental function. Future studies that explore these relationships at the protein level, employing physiologically relevant concentrations, may provide insights into mechanistic interactions between PFAS and human trophoblast dysfunction.

Data availability

Supplementary data are available at https://doi.org/10.5061/dryad.2z34tmprb.

Author contributions

All authors contributed to the study conception and design. Experiments were performed by H.C., M.K., D.G., S.B., U.L., A.S., and J.R. Statistical analyses were conducted by H.C., J.L., D.G., and J.R. Interpretation of the data was performed by H.C., D.G., S.A., S.G., T.W., S.F., and J.R. The initial draft of the manuscript was written by H.C. and J.R., which was edited by H.C., D.A., S.G., T.W., S.F., and J.R. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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