Lipogenic Potency of Individual Perfluorinated Alkyl Acids (PFAAs) and Persistent Organic Pollutant (POP) Mixtures at Human Blood-Based Exposure Levels on Adipogenesis in 3T3-L1 Cells

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Lipogenic potency of individual perfluorinated alkyl acids (PFAAs) and persistent organic pollutant (POP) mixtures at human blood-based exposure levels on adipogenesis in 3T3-L1 cells

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Abstract

**Background:** In recent decades, the incidence of metabolic disorders has increased internationally. This increase has been linked to exposure to persistent organic pollutants (POPs) but little is known about the metabolic effects of realistic human exposure mixtures at relevant concentrations.

**Objectives:** In this study we tested if POPs, representing real life exposure profiles and concentrations, were able to disrupt development and functions of adipose tissue in a direct way.

**Methods:** The lipogenic potency of a POP mixture modelled on levels found in human blood as detected in the Scandinavian population was assessed. The Total mixture comprises 29 compounds divided over three groups: chlorinated (Cl), brominated (Br), and perfluorinated compounds (PFAA). Individual PFAA chemicals, the Total mixture, and sub-mixtures (Cl, Br, PFAA, Cl + Br, Cl + PFAA, and Br + PFAA) at five (× 1/10, × 1, × 50, × 100, and × 500) human blood levels were tested in an optimised high content analysis (HCA) 3T3-L1 adipogenesis assay.

**Results:** Individual PFAAs; perfluorohexanesulfonic acid (PFHxS), perfluorooctanesulfonic acid (PFOS), perfluorononanoic acid (PFNA), and perfluoroundecanoic acid (PFUnDA) promoted lipid accumulation in 3T3-L1 cells. The Total mixture, and the Cl, PFAA, Cl + Br, and Cl + PFAA sub-mixtures, promoted adipogenic differentiation and lipid accumulation. Increased lipid accumulation promoted adipose tissue expansion.

**Conclusions:** To the authors knowledge, this is the first *in vitro* bioassay study assessing the adipogenic effects of POP mixtures modelled on real-life human exposure levels. The findings highlight that such exposures may alter adipose tissue development and function, thus potentially playing a role in the globally increasing escalation of metabolic disorders.
1 Introduction

Persistent organic pollutants (POPs) maintain a strong structural stability that enables them to persist for a long time in the environment and accumulate in biota (WHO/UNEP, 2013). Consequently, we are exposed to these chemicals and their mixtures via skin contact, our diet (including drinking water) and the air we breathe (Braun et al., 2016; Connolly, 2009). Exposure to POPs has been associated with an increased risk of reproductive impairments, neurodevelopmental dysfunctions, and cancers (WHO/UNEP, 2013). More recently, exposure to ubiquitous chemicals has also been counted as a major contributor to the global epidemic of metabolic disruption. Review of data from 1988 to 2014 report that child growth and obesity are linked with exposures to PCBs, organochlorine pesticides (OCPs), and perfluorinated chemicals (Heindel et al., 2017; Karlsen et al., 2017).

As more synthetic chemicals have been introduced to our society, many have shown metabolic disrupting effects via adipose tissue. White adipose tissue is an endocrine organ which stores excessive energy in the form of triacylglycerol, a neutral lipid. This lipid is held within lipid vacuoles in adipocytes, resulting in body fat accumulation. White adipose tissue has been identified as a target of metabolic disruption by POPs (Heindel et al., 2019; Heindel et al., 2015; Neel and Sargis, 2011; Regnier and Sargis, 2014). The investigation of POPs and their effects on adipose tissue development is necessary to help understand how environmental factors promote the pathogenesis of obesity and metabolic complications (Newbold et al., 2008). In particular, there is a need to study the metabolic disrupting effects of these chemicals at levels and mixtures representing real life exposure. Maternal serum and breast milk levels of PCBs and organochlorine pesticides DDE have been linked to the subsequent development of obesity in girls (Tang-Péronard et al., 2014). Hence, a significant body burden of POPs in adipose
tissue may lead to continuous local exposure and disruption of adipose biology. Adult studies have shown that body mass index (BMI) is inversely related to PCB blood concentrations and that weight loss can result in the release of PCBs into the circulation (Agudo et al., 2009; Dirinck et al., 2015; Hue et al., 2007). During weight loss, the breakdown and decrease of body fat can lead to the release and resulting increase in organochlorine pollutant plasma concentrations, indicating that adipose tissue can function as an in vivo exposure source of POPs (Bräuner et al., 2011; De Roos et al., 2012).

This study aimed to investigate the potential for POPs and their mixtures to disrupt adipogenesis at human relevant exposure levels, providing insight into alternative mechanisms by which POPs may be involved in the disruption of adipocyte development and pathogenesis of obesity and metabolic disorders.

To simulate real-life POP mixture exposure profiles, we utilised a synthetic mixture of 29 POPs (Table 1) which was designed on the mean human blood levels of these POPs within the Scandinavian population (Berntsen et al., 2017). These 29 POPs include three chemical groups: chlorinated chemicals (Cl) consisting of polychlorinated biphenyls (PCBs) and OCPs, brominated chemicals (Br), and perfluorinated compounds (PFAA). A Total mixture and 6 sub-mixtures containing the same concentration of chlorinated and brominated (Cl + Br), chlorinated and perfluorinated (Cl + PFAA), or chlorinated (Cl), brominated (Br), or perfluorinated (PFAA) compounds only were constructed by Berntsen et al., 2017. The sub-mixtures were created to enable the assessment of effects upon adding or removing one or more chemical groups.
Table 1, Overview of the 29 different POPs constituting the Total mixture for *in vitro* evaluation.

For a complete description of selection of compounds and their individual concentration, see Berntsen et al., 2017.

| Chlorinated | Brominated | Perfluorinated |
|-------------|------------|----------------|
| PCB         | OCP        | BFR            |
| PCB 28      | p,p'-DDE   | PBDE 47        | PFHxS |
| PCB 52      | HCB        | PBDE 99        | PFOS  |
| PCB 101     | α-Chlordane| PBDE 100       | PFOA  |
| PCB 118     | Oxychlordane| PBDE 153     | PFNA  |
| PCB 138     | trans-Nonachlor| PBDE 154   | PFDA  |
| PCB 153     | α-HCH      | PBDE 209       | PFUnDA|
| PCB 180     | β-HCH      | HBCD           |
|             | γ-HCH      |                |
|             | (Lindane)  |                |
|             | Dieldrin   |                |
Individual PFAAs have previously been reported as biologically active in other *in vitro* assays assessing obesogenic potential using 3T3-L1 cells (Watkins et al., 2015). However, these effects were confirmed at higher concentrations than the human relevant levels in our mixtures. In addition, two of the compounds which are included in our mixtures, PFUnDA and PFDA, have not been previously assessed. We have also previously shown that our PFAA mixture is capable of inducing higher secretion of GLP-1, a gut hormone which is involved in obesity and satiety signalling pathways, than the Cl and Br mixtures (Shannon et al., 2019). Consequently, we decided to also assess the adipogenic potential of the PFAA chemicals individually and at the realistic exposure levels of the human exposure study (Berntsen et al., 2017).

The 3T3-L1 murine preadipocyte cell line has previously been used to elucidate the effects of individual POPs (at high concentrations) on the development of adipose tissue (Heindel, 2019; Regnier and Sargis, 2014). In the current study, we aimed to utilise a 3T3-L1 adipogenesis high content analysis (HCA) protocol to study the effects of real-life POP exposure on adipogenesis.  

### 2 Materials and Methods

#### 2.1 Reagents

All PBDEs, PCBs and other organochlorines were originally purchased from Chiron AS (Trondheim, Norway). All PFAAs were obtained from Sigma-Aldrich (St. Louis, MO, USA) except perfluorohexanesulfonic acid (PFHxS) which was from Santa Cruz (Dallas, US). The anti-fungal reagent, hexabromocyclododecane (HBCD), phosphate buffered saline (PBS) and dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (Dorset, UK). The nuclear stain DAPI and all cell culture reagents were supplied by Life Technologies (Paisley, UK) unless otherwise stated. The reference standard rosiglitazone (ROSI) and lipid content dye
Nile Red were obtained from Premier Scientific (Belfast, UK). All other reagents were standard laboratory grade.

2.2 Mixtures of persistent organic pollutants (POPs)

The POP mixtures tested in this study were designed and premade by the Norwegian University of Life Sciences, Oslo (Berntsen et al., 2017). Seven mixtures were tested in the current study: A Total mixture, containing 29 compounds, in addition to Cl, Br, PFAA, Cl + Br, Cl + PFAA, and Br + PFAA sub-mixtures. Six individual PFAAs were also tested including, PFHxS, perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnDA), at concentrations equivalent to the mixture concentrations. Five concentrations of mixtures and individual compounds were used representing the widely varied human exposure scenarios, including: × 1/10, × 1, × 50, × 100, and × 500 human blood relevant levels. Table 1 presents an overview of the 29 individual compounds comprising the 7 mixtures and further extensive information on the mixture development can be found in Berntsen et al. (2017), whereas the concentrations of the POPs in the mixtures are available in Supplementary Table 1.

2.3 Cell Culture

The 3T3-L1 preadipocyte murine cell line (catalogue number: 86052701) capable of differentiation into mature adipocytes under hormonal stimulation was supplied by Thermo Scientific (UK). Cells were grown in 75 cm² tissue culture flasks (Nunc, Roskilde, Denmark) at 37°C with 5% CO₂ and 95% humidity. Culture medium containing Dulbecco’s Modified Eagle Medium (DMEM), Glutamax™, 10% foetal bovine serum (FBS) and 1% penicillin and streptomycin (50 U/ml) was replaced every two days to ensure sufficient nutrient supply and anti-microbial capacity. Subculture was required when cells reached 90% confluence. Cells between passage number 7 to 17 were used for this assessment.
2.4 Adipocyte differentiation and POP exposure

The 3T3-L1 cells were seeded into 96-well black-wall plates at 2,000 cells per well in 100 µl preadipocyte medium (PM (DMEM Glutamax™ supplemented with 10% FBS and 2% Pen/Strep)). After 48 h, cells were exposed to treatments in differentiation medium (DM) that consisted of PM and differentiation inducing mixture (DIM) for 48 h. The DIM consisted of 43 nM insulin, 100 nM dexamethasone, and 25 µM of 3-isobutyl-1-methylxanthine (IBMX). The solvent control (SC) containing 0.2% DMSO, positive control ROSI (at 0.4 µg/ml), POP mixtures, and individual PFAAs were applied to cells. All treatments were made with a final (DMSO) concentration of 0.2% in DM. After 48 h exposure with treatments containing DM, the wells were emptied and 200 µl of insulin medium (IM) (PM with 43 nM insulin) was added and plates incubated for 48 h on Day 5. On Day 7 the IM was replaced by PM and the plates read 48 h later on Day 9. A preadipocyte control group was included where cells were maintained in PM without differentiation induction for 8 days. The entire protocol is shown in Figure 1.

Figure 1. Adipogenesis protocol. The adipocyte differentiation protocol for cell treatment.
2.5 MTT assessment

Metabolic activity is essential for cellular proliferation, differentiation, and lipid metabolism. The metabolic activity of 3T3-L1 cells exposed to the six individual PFAAs, Total mixture, Cl, Br, Cl + Br, Cl + PFAA, and PFAA + Br, mixtures, was assessed by thiazolyl blue tetrazolium bromide (MTT) assay on Day 5 (immediately after 48 h exposure) and Day 9 after full adipogenic development. The test on Day 5 aims to assess the immediate effects of POP exposure on metabolic activity of 3T3-L1 cells. Apoptotic and necrotic cells lose the capacity to reduce MTT to formazan. Thus, the immediate assessment of MTT levels can examine whether POP exposure induced any cellular damage. On Day 9, the MTT readout were assessed to measure the metabolic activity of exposed and fully differentiated cells. Exposed cells were washed with 200 µl PBS once, and 50 µl MTT solution (2 mg/ml stock in PBS diluted 1:6 in basal media) was added into each well and incubated at 37°C in 5% CO₂ and 95% humidity. After 4 hours, the MTT solution was discarded, and 200 µl of preheated DMSO solution added to each well and incubated at 37°C for 10 minutes in a plate shaker. Optical density was measured at 570 nm with a reference wavelength at 630 nm. Results were presented as percentage of the solvent control, set to 100%.

2.6 High content analysis

High content analysis is a useful tool to collect multiple markers to assess the cellular responses to exposures. By applying fluorescent dyes, nuclear markers including cell number, nuclear area, nuclear intensity, and intracellular lipid content were measured in the current study. Cell number refers to numbers of nuclei, indicating cell proliferation, growth inhibition, or cell death induced by treatment. Nuclear area and intensity give further information about the cellular health status. Changes in nuclear area and intensity can indicate cells undergoing a pre-lethal phase. Increased nuclear area and decreased nuclear intensity are more commonly
observed in necrotic cells, while a small nuclear area is more related to apoptosis (Doonan and Cotter, 2008). However, cell reconstruction occurs during adipogenesis, which gives smaller and denser nuclei to allow lipid droplet enlargement. Thus, in the case of 3T3-L1 cell adipogenesis, decreased nuclear area and increased nuclear intensity may occur without pre-lethal effects. Intracellular lipid content represents the level of adipogenic differentiation and lipid accumulation. Differentiated 3T3-L1 cells were stained and read on Day 9. Plates were washed once with 200 µl PBS, and fixed with 100 µl formalin (approximately 4% formaldehyde) for 10 min at room temperature (RT). Cells were then washed twice with 200 µl PBS, and stained with 100 µl of 300 nM DAPI solution for 10 min at RT. After DAPI staining, two further washes in PBS were performed. A 0.25 µg/ml Nile Red working solution was made by dilution of the 250 µg/ml Nile Red DMSO stock solution in PBS, and was added to each well for 20 min. The Nile Red solution was thereafter discarded and 100 µl of PBS added to the wells prior to sealing the plates with black film to protect fluorescent staining from light. The results were read using the CellInsightTM NXT High Content Screening (HCS) platform (Thermo Fisher Scientific, UK). Images were captured at ×10 objective magnification and selected filters: DAPI (Ex/Em 350/461 nm) and Nile Red (Ex/Em 554/576 nm) (Figure 2).
Figure 2, High content images of fluorescent stained 3T3-L1 cells on Day 9. A) undifferentiated 3T3-L1 cells that treated with solvent control DMSO; B) differentiated 3T3-L1 cells treated with 0.4 µg/ml rosiglitazone. The typical HCA micrographs are shown with nuclei (blue, stained by Hoechst 33342) and neutral lipid content (red, stained by Nile Red) with X 10 objective magnification, scale bars = 100 µ.

2.7 Data analysis

Exposures were carried out in triplicate wells and three independent exposures were performed (n=3). Data collected from the HCS platform was processed with Excel and Graphpad PRISM software version 6.0 (San Diego, CA). All values shown are expressed as mean ± standard error of measurement (SEM) of three independent exposures for the compounds tested. Data is expressed as a percentage of the relevant solvent control for each parameter and analysed by one-way ANOVA followed by Dunnett’s procedure for multiple comparisons; the mean concentrations were tested for significant difference at the 95% confidence level. Significant effects are represented by \( P \leq 0.05 (*) \), \( P \leq 0.01 (**) \) and \( P \leq 0.001 (***) \). Data for lipid content results were analysed by calculating the response when compared with the positive control.
3 Results

3.1 Metabolic activity in POPs treated 3T3-L1 cells by MTT

MTT metabolic activity provides information about the cellular condition based on the levels of mitochondrial reductase activity. On Day 5, only PFHxS and PFOA significantly reduced metabolic activity (Fig. 3A). For PFHxS this occurred from x 100 blood levels and higher. For PFOA the effect was already visible at 1/10 blood levels. On Day 9 only PFUnDA at × 500 blood levels significantly reduced MTT activity. Numerical tabulated data is available in Supplementary Table 2.

Figure 3. MTT metabolic activity of differentiated 3T3-L1 cells after exposure to individual PFAAs. Tested after differentiation on A) Day 5 and B) Day 9 to individual PFAAs at x 1/10, x 1, x 50, x 100 and x 500 of blood levels. Data is expressed as a percentage of solvent control (0.2% DMSO) set at 100% (dotted line) for each parameter; mean ± SEM, n = 3. p ≤0.05 (*), P ≤0.01 (**) and P ≤0.001 (***) represent significant change in metabolic activity.

On Day 5, the Total mixture reduced MTT metabolic activity at x 1/10, x 100 and x 500 blood level (Fig. 4A). The PFAA mixture decreased MTT activity at the highest concentration (× 500 blood level) (Fig. 4A). On Day 9, none of the POP mixtures (Fig. 4B) significantly changed
the metabolic activity (Fig. 4B). Numerical tabulated data is available in Supplementary Table 3.

Figure 4. MTT metabolic activity of 3T3-L1 cells after exposure to POP mixtures. Tested on A) Day 5 and B) Day 9, after exposure to POP mixtures at x 1/10, x 1, x 50, x 100 and x 500 of blood levels. Data is expressed as a percentage of solvent control (0.2% DMSO) set at 100% (dotted line) for each parameter; mean ± SEM, n = 3. P ≤0.05 (*), P ≤0.01 (**) and P≤0.001 (***) represent significant change in metabolic activity.

3.2 Cell number and nuclear morphology

MTT readout revealed a decrease in metabolic activity for certain exposures. HCA nuclear markers are useful to provide additional information to explain the change of metabolic activity. Cell number was used to check for loss of cells. On Day 9, no significant changes in cell number were observed for the six individual PFAAs. Similarly, the Total mixture did not affect cell number. However, the Cl + Br mixture led to a significant cell number reduction at × 50 blood level (P ≤ 0.05) (Fig. 5). The Cl + PFAAs mixture induced a significant reduction in cell number at x 50 (P ≤ 0.001) and × 100 blood levels (P ≤ 0.05) (Fig. 5).
Figure 5. Cell number of 3T3-L1 cells. Tested after differentiation on Day 9. Data is expressed as a percentage of solvent control (0.2% DMSO) for each parameter; mean ± SEM, n = 3. P ≤ 0.05 (*), P ≤ 0.01 (**) and P ≤ 0.001 (***) represent significant reducing effects.

Change in nuclear area or nuclear intensity could be indicative of cell death processes. No significant changes in these parameters were observed for any of the individual PFAAs. Similarly, neither the Total mixture, nor any of the sub-mixtures induced significant nuclear morphological change. Numerical tabulated data is available in Supplementary Table 2 and 3.

3.3 Lipid accumulation

The intracellular lipid accumulation present in differentiated adipocytes, was assessed by measuring the intensity of fluorescent dye stained neutral lipid content. On Day 9, PFHxS, PFNA, and PFUnDA induced significant lipid accumulation at higher concentrations (× 100 and/or × 500). PFOS induced significant lipid accumulation at × 1/10 blood levels but failed
to increase lipid accumulation at higher concentrations tested (Fig. 6 and Supplementary Table 4).

Figure 6. PFAAs modulate lipid accumulation during 3T3-L1 adipocyte differentiation in a complex dose-response pattern. Increased lipid content in 3T3-L1 cells after exposure to PFOS, PFHxS, PFNA, and PFUnDA at x 1/10, x 1, x 50, x 100 and x 500 of blood levels. Data is expressed as a percentage of positive control (rosiglitazone at 0.4 µg/ml) set at 100% (dotted line) for each parameter; mean ± SEM, n = 3. P ≤0.05 (*), P ≤0.01 (**) and P ≤0.001 (***)) represent significant change in lipid content that compared to solvent control (set as 0%).

The Total mixture and all the sub-mixtures, except the Br and PFAA + Br, induced a significant lipid content increase at different concentrations (Supplementary, Table 5). The Total, PFAA, and Cl + PFAA mixtures presented a non-monotonic lipid content profile (Figure 7). Cl mixture gave significant lipid accumulation at × 1/10 blood level, whereas the Cl + Br mixture promoted significant lipid increase at × 1/10 and ×100 blood levels.
Figure 7. POP mixtures modulate lipid accumulation in 3T3-L1 adipocyte differentiation in complex dose-response pattern. Lipid accumulation in 3T3-L1 after treatment of seven POP Mixtures at x 1/10, x 1, x 50, x 100, and x 500; Data is expressed as a percentage of positive control (rosiglitazone at 0.4 µg/ml) set at 100% (dotted line) for each parameter; mean ± SEM, n = 3. P ≤ 0.05 (*), P ≤ 0.01 (**) and P ≤ 0.001 (***) represent significant change in lipid content that compared to solvent control (set as 0%).
4 Discussion

Since the ‘environmental obesogen hypothesis’ announced by Grun and Blumberg (2006), extensive studies have been carried out to demonstrate the link between obesity and metabolic disorders induced by POPs (Sargis et al., 2009; Regnier and Sargis, 2014; vom Saal et al., 2012; Watkins et al., 2015). In the current study, the lipogenic potential of human relevant POP mixtures and the PFAA group of chemicals that represent Scandinavian exposure concentrations have been tested. The dietary pattern and occupational environment can also lead to higher exposure levels (Fu et al., 2016; Krüger et al., 2007; Laird, Goncharov and Chan, 2013). In human and laboratory studies, the importance of low dose effects and nonmonotonic dose responses have been highlighted, therefore it is important to assess a wide range of exposures for the effective risk assessment of chemicals (Lee et al., 2010; Vandenberg et al., 2012). The blood levels of POPs in the US population have been reported as ten to hundred times higher than the European population (Schecter et al., 2005). Thus, in addition to the concentration levels found in the Scandinavian population, a wider range of concentrations (from $1/10$ to $500 \times$ blood levels) were tested to mimic different exposure scenarios.

In 3T3-L1 cells, adipogenic differentiation can be initiated after the induction with insulin, IBMX, and dexamethasone treatment. Increased mitochondrial biogenesis, and promoted adenosine triphosphate (ATP) and dihydronicotinamide-adenine dinucleotide phosphate (NADPH) production have been identified during adipogenesis (Liu et al., 2014). The metabolism switches from glycolysis to oxidative phosphorylation during differentiation, which is followed by lipogenesis and changes in cell phenotype (Xu et al., 2013). MTT is an assay that is performed routinely to assess cell viability but is also a useful technique to test metabolic activity. At the start of differentiation, represented by cells on Day 5, the change in MTT metabolic activity can provide information about the cellular metabolic status that impact
the differentiation progress at the point of mitochondrial bioenergetic transition from anaerobic to aerobic metabolism. A decrease in MTT activity may suggest impaired adipogenesis. Based on our observations, the magnitude of MTT metabolic activity reduction by the different PFAAs can be ranked as follows: PFOA > PFHxS > PFOS = PFDA = PFNA = PFUnDA. The magnitude of MTT metabolic activity reduction by the different mixtures can be ranked as follows: Total mixture > PFAA > Cl = Br = Cl + PFAA = Cl + Br = PFAA + Br. The PFAA sub-mixture showed more potent effect on metabolic activity than the Cl and Br sub-mixtures. When PFAA was combined with Cl or Br chemicals, Cl + PFAA and PFAA + Br sub mixtures also induced less MTT change than the PFAA sub-mixture. However, the Total mixture was more potent than PFAA, which suggested the combination of PFAA, Cl, and Br chemicals together had greater effect on MTT reduction (see Supplementary Table 3).

By looking at the changes of MTT metabolic activity after differentiation on Day 9, the effects of treatments on metabolic activity and ATP production at the end of differentiation can be assessed. But here none of the pollutants (except high PFUnDA) had any effect on MTT activity. This seems to indicate the metabolic effect of POPs, if any, is more pronounced at the start of differentiation. Between Day 5 and Day 9, cells were treated with lipogenesis promoting and nutrient supplying insulin medium and basal medium only. Thus, this may result in the recovery and growth of cells after withdrawal of pollutants. Enhanced mitochondrial activity during lipogenesis might also explain the restored metabolic activity at Day 9, compared to Day 5.

DAPI is a DNA binding fluorescent dye which stains nuclei, thus giving information on cell number, nuclear area, and nuclear intensity (Filippi-Chiela et al., 2012). These nuclear markers were only assessed on Day 9 to investigate the cellular health condition after POP exposure.
and full differentiation. Cl + Br and Cl + PFAA mixtures induced a significant decrease in cell number. No changes in cell number were seen amongst all other POP exposures at any concentration. In the current study the POP exposures failed to induce any significant changes on nuclear morphological markers in the 3T3-L1 cells, thus indicating absence of direct cell death induction. Combining with MTT results, the changes in cell number after exposure to the Cl + Br and Cl + PFAA mixtures may impact the insulin-driven processes that down-regulate clonal expansion.

By looking at cell number, nuclear area, nuclear intensity, and MTT results, other POP exposures did not induce significant cell population changes or cellular damage in differentiated 3T3-L1 cells. However, the HCA assessment was not applied on Day 5. Thus, the current study was unable to track the changes in cell number between two end points.

The changes in lipid content of the 3T3-L1 cells were expressed as a percentage of the positive control ROSI which is a selective peroxisome proliferator-activated receptor gamma (PPARγ) agonist. PPARγ is essential and sufficient to induce adipogenesis in vitro, and embryonic adipogenesis in vivo (Chiarelli and Di Marzio, 2008; Wang et al., 2015). For relative comparison to human effects levels, the concentration of ROSI (0.4 µg/ml) used in this study was in the range of pharmaceutical levels (GlaxoSmithKline, 2019).

When tested, some PFAAs showed lipogenic effects after adipogenic differentiation. PFHxS, PFOS, and PFNA induced significant lipid content increase in 3T3-L1 cells with the lowest observed effect concentrations at 80, 200, and 40 µM respectively (Watkins et al., 2015). In our study, PFHxS, PFOS, and PFNA induced observable effects at lower levels of 390.46 nM, 415.22 nM, 10.93 nM. Contradictory to previous studies (Watkins et al., 2015; Yamamoto,
Yamane, Oishi and Kobayashi-Hattori, 2014), PFOA did not increase lipid content on Day 9. The reduction in MTT results on Day 5 for PFOA were observed at all tested levels. Hence, the reduction in metabolic activity may be the reason of failure of lipid accumulation in the later differentiation stage. PFUnDA has not previously been tested in the 3T3-L1 cell line, but showed the most potent lipogenic property in the HCA readout. PFUnDA-induced lipid accumulation occurred at 3.368 nM, but not at the higher concentration of 16.84 nM. The decreased MTT activity on Day 9 observed at 16.84 nM (corresponding to $\times 500$ blood levels) may suggest that the disrupted metabolic activity impaired the aerobic metabolism and lipid accumulation. In hPPAR\(\gamma\)-LBD luciferase reporter transfected HepG2 cells, all six individual PFAAs tested in this study have been shown to activate hPPAR\(\gamma\) in a dose-dependent manner (Zhang et al., 2014). Individual PFAAs mimic ROSI promoted differentiation and lipid accumulation, indicating that these POPs could possibly act as PPAR\(\gamma\) agonists.

Strikingly, several mixtures increased lipid content already at concentrations equivalent to 1/10 human blood levels. The Total mixture showed the most potent lipogenic effect at this concentration. For the Cl mixture, this corresponds to a sum of Cl chemicals of about 330 pM. Previously, individual PCBs at 1 \(\mu\)M, or combinations thereof at 1 \(\mu\)M promoted lipid accumulation in 3T3-L1 cells (Ferrante et al., 2014). PCB-118 and 138 have been shown to promote adipogenesis of 3T3-L1 \textit{in vitro}, and increase adipogenesis \textit{in vivo} (Kim et al., 2016a). \(p'p\)-DDE, the chlorinated compound with highest concentration in our mixture, as well as oxy-chlordane, \(\gamma\)-HCH, and dieldrin also showed adipogenic potential in adipogenesis studies (Howell and Mangum, 2011; Kanayama et al., 2004; Kim et al., 2016b; Mangum, Howell and Chambers, 2015). \(\alpha\)-chlordane, \(\alpha\)-HCH, and \(\beta\)-HCH have not been assessed in adipose biology previously, but showed adipogenic and metabolic disrupting properties in \textit{in vivo} studies (Guo et al., 2014; Lopez-Espinosa et al., 2011). The PFAA mixture lead to a non-monotonic response,
but the highest lipid accumulation was induced already at $\times 1/10$ blood levels, corresponding to a concentration for the total sum of PFAA of 5.53 nM. For the Cl + PFAA mixture, significant lipid accumulation was observed at $\times 1/10$, $\times 1$, $\times 50$, and $\times 500$ blood levels. A drop in lipid content occurred at $\times 50$ and $\times 100$ blood levels, and accompanied a drop in cell number.

The Br mixture did not induce any significant lipid accumulation at any tested concentrations. BDE-47 showed significant differentiation, lipid accumulation promoting, PPAR\(\gamma\) binding, and expression promoting effects in 3T3-L1 cells at 3 nM (Kamstra et al., 2014; Kassotis, Hoffman, and Stapleton, 2017). The concentration of BDE-47 in the current study was 0.89 nM at $\times 500$ blood levels, which is lower than the adipogenic concentration in the previous study (Kamstra et al., 2014). BDE-99, BDE-209, and HBCD did not induce any change in 3T3-L1 cells up to 10 \(\mu\)M (Kassotis, Hoffman, and Stapleton, 2017). A mixture of BDE congeners (also contains BDE-47, 100, 153, and 209) showed adipogenesis enhancement in the 3T3-L1 adipogenesis model accompanied by PPAR\(\gamma\) activation at 25.5 \(\mu\)M (Tung et al., 2014). The sum of Br chemicals in our exposures is 4.905 nM even at $\times 500$ blood levels. The low concentrations of Br chemicals in our mixture may explain the negative results in lipid accumulation. However, rodent model studies revealed that maternal exposure to low levels of BED-47 promoted obesity development with significant lipid metabolism disruption in off-springs (Gao et al., 2019; Wang et al., 2018). These in vivo results suggested that low dose exposure in the early life stage may play critical roles in obesity development in the later stage. For PFAA + Br the effects on lipid accumulation are of a similar magnitude as for the PFFA or Br sub-mixtures alone, indicating an absence of additive effects. On the other hand, Cl + Br mixture induced significant lipid content increase at $\times 1$ and higher blood levels, which suggested Br chemicals may promote the lipogenic capacity of Cl chemicals.
In the current study, increased intracellular lipid accumulation after exposure to human relevant concentrations of individual PFAAs, and POP mixtures were observed. Certain POP exposures impacted metabolic activity in the early stage of adipogenesis, but not the later. However, POP-enhanced adipocyte formation and lipid accumulation may induce excessive body fat content (Grün et al., 2006). Thus, the current study findings suggest that the low dose effects of POPs on adipose tissue needs to be considered in evaluating the metabolic health effects of POPs.

**5 Conclusion**

In this study, human relevant POP mixture exposures induced significant lipid accumulation in 3T3-L1 cells. Increased adipogenesis and lipid accumulation may lead to impaired adipose tissue function and excessive fat storage in the body. Therefore, real-life POP mixture exposure at low doses may induce metabolic effects in adipose tissue. The individual chemical PFOS, and Cl and PFAA sub-mixtures were the main drivers at low concentrations. Combinatorial effects were observed when Cl chemicals were combined with PFAAs, and Cl chemicals were combined with Br chemicals, but not in the combination of the PFAA + Br sub-mixture. The total mixture induced significant lipid increases at all tested concentrations, with stronger induction than the sub-mixtures. The combinational effect suggests compounds collectively induce effects at lower concentrations than what has previously been observed for the single compounds. This study therefore highlights the importance of assessing real life exposure mixtures for effective risk assessment.

**DECLARATION OF CONFLICTING INTERESTS**

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

All data generated or analysed during this study are included in this article (and its supplementary information files).

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Figures

Figure 1

Adipogenesis protocol. The adipocyte differentiation protocol for cell treatment.
Figure 2

High content images of fluorescent stained 3T3-L1 cells on Day 9. A) undifferentiated 3T3-L1 cells that treated with solvent control DMSO; B) differentiated 3T3-L1 cells treated with 0.4 μg/ml rosiglitazone. The typical HCA micrographs are shown with nuclei (blue, stained by Hoechst 33342) and neutral lipid content (red, stained by Nile Red) with X 10 objective magnification, scale bars = 100 μ.

Figure 3
MTT metabolic activity of differentiated 3T3-L1 cells after exposure to individual PFAAs. Tested after differentiation on A) Day 5 and B) Day 9 to individual PFAAs at x 1/10, x 1, x 50, x 100 and x 500 of blood levels. Data is expressed as a percentage of solvent control (0.2% DMSO) set at 100% (dotted line) for each parameter; mean ± SEM, n = 3. p ≤0.05 (*), P ≤0.01 (**) and P ≤0.001 (***) represent significant change in metabolic activity.

Figure 4

MTT metabolic activity of 3T3-L1 cells after exposure to POP mixtures. Tested on A) Day 5 and B) Day 9, after exposure to POP mixtures at x 1/10, x 1, x 50, x 100 and x 500 of blood levels. Data is expressed as a percentage of solvent control (0.2% DMSO) set at 100% (dotted line) for each parameter; mean ± SEM, n = 3. P ≤0.05 (*), P ≤0.01 (**) and P ≤0.001 (***) represent significant change in metabolic activity.
Figure 5

Cell number of 3T3-L1 cells. Tested after differentiation on Day 9. Data is expressed as a percentage of solvent control (0.2% DMSO) for each parameter; mean ± SEM, n = 3. P ≤0.05 (*), P ≤0.01 (**) and P ≤0.001 (***)) represent significant reducing effects.
Figure 6

PFAAs modulate lipid accumulation during 3T3-L1 adipocyte differentiation in a complex dose-response pattern. Increased lipid content in 3T3-L1 cells after exposure to PFOS, PFHxS, PFNA, and PFUnDA at x 1/10, x 1, x 50, x 100 and x 500 of blood levels. Data is expressed as a percentage of positive control (rosiglitazone at 0.4 μg/ml) set at 100% (dotted line) for each parameter; mean ± SEM, n = 3. P ≤0.05 (*), P ≤0.01 (**) and P ≤0.001 (***) represent significant change in lipid content that compared to solvent control (set as 0%).
Figure 7

POP mixtures modulate lipid accumulation in 3T3-L1 adipocyte differentiation in complex dose-response pattern. Lipid accumulation in 3T3-L1 after treatment of seven POP Mixtures at x 1/10, x 1, x 50, x 100, and x 500; Data is expressed as a percentage of positive control (rosiglitazone at 0.4 μg/ml) set at 100% (dotted line) for each parameter, mean ± SEM, n = 3. P ≤0.05 (*), P ≤0.01 (**) and P ≤0.001 (***) represent significant change in lipid content that compared to solvent control (set as 0%).

Supplementary Files

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