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Legacy and alternative per- and polyfluoroalkyl substances (PFAS) alter the lipid profile of HepaRG cells

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ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals used in various industrial and consumer products. They have gained attention due to their ubiquitous occurrence in the environment and potential for adverse effects on human health, often linked to immune suppression, hepatotoxicity, and altered cholesterol metabolism. This study aimed to explore the impact of ten individual PFAS, 3 H-perfluoro-3-[(3-methoxypropoxy) propanoic acid] (PMPP/Adona), ammonium perfluoro-(2-methyl-3-oxahexanoate) (HFPO-DA/GenX), perfluorobutanoic acid (PFBA), perfluorobutanesulfonic acid (PFBS), perfluorodecanoic acid (PFDA), perfluorohexanoic acid (PFHxA), perfluorohexanesulfonate (PFHxS), perfluorononanoic acid (PFNA), perfluorooctanoic acid (PFOA), and perfluorooctanesulfonic acid (PFOS) on the lipid metabolism in human hepatocyte-like cells (HepaRG). These cells were exposed to different concentrations of PFAS ranging from 10 μM to 5000 μM. Lipids were extracted and analyzed using liquid chromatography coupled with mass spectrometry (LC- MS-QTOF). PFOS at 10 μM and PFOA at 25 μM increased the levels of ceramide (Cer), diacylglycerol (DAG), N-acylethanolamine (NAE), phosphatidylcholine (PC), and triacylglycerol (TAG) lipids, while PMPP/Adona, HFPO-DA/GenX, PFBA, PFBS, PFHxA, and PFHxS decreased the levels of these lipids. Furthermore, PFOA and PFOS markedly reduced the levels of palmitic acid (FA 16.0). The present study shows distinct concentration-dependent effects of PFAS on various lipid species, shedding light on the implications of PFAS for essential cellular functions. Our study revealed that the investigated legacy PFAS (PFOS, PFOA, PFBA, PFDA, PFHxA, PFHxS, and PFNA) and alternative PFAS (PMPP/Adona, HFPO-DA/GenX and PFBS) can potentially disrupt lipid homeostasis and metabolism in hepatic cells. This research offers a comprehensive insight into the impacts of legacy and alternative PFAS on lipid composition in HepaRG cells.

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) constitute a group of synthetic chemicals extensively used across industrial- and consumer sectors, with thousands of individual chemicals on the market today and hundreds actively used in commercial applications, inter alia, in consumer products (Buck et al., 2021; Dewapriya et al., 2023; Glüge et al., 2020; Herzke et al., 2012). PFAS are subject to increasing concern due to their persistence, environmental mobility, and potential to induce adverse health effects such as immune suppression, hepatotoxicity, and altered lipid metabolism. PFAS have been repeatedly found to be positively associated with increased blood cholesterol concentrations and, in some cases, triacylglycerol levels. Multiple epidemiological studies have reported changes in the levels of various blood lipids to be associated with PFAS exposure (Aker et al., 2023; Canova et al., 2020; Nelson et al., 2010; Steenland et al., 2009). Animal data also support the notion that PFAS alter lipid metabolism (Das et al., 2017; Pfohl et al., 2020). These effects of PFAS have been reported to be mediated by nuclear receptors

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(NRs), dominated mainly by peroxisome proliferator-activated receptor alpha (PPARα) and, to a lesser extent, peroxisome proliferator-activated receptor gamma (PPARγ), constitutive androstane receptor (CAR), and pregnane X receptor (PXR) (Attema et al., 2022; Bjork et al., 2011; Das et al., 2017; Rosen et al., 2017; Vanden Heuvel et al., 2006). These NRs play a diverse role in regulating lipid and energy metabolism (Varga et al., 2011; Wagner et al., 2005).

The directions of lipid changes are often opposed between animal and human studies (Fragki et al., 2021). Therefore, we need to better understand PFAS's impact on lipid metabolism. Investigating PFAS's effects on lipid profiles and lipid species is essential for obtaining new insights into the mode of action of PFAS toxicity.

In recent PFAS health risk assessments, an increase in the serum cholesterol concentration has emerged as a critical endpoint, along with decreased immune response and hepatotoxicity (EFSA CONTAM Panel, 2018, 2020). These assessments underscore the significance of comprehending PFAS-induced alterations in lipid metabolism as a crucial component in determining the potential health risks associated with exposure to these substances. A recurring theme within the regulatory domain is the potential link between PFAS-induced blood lipid perturbations and human disease outcomes (EFSA CONTAM Panel, 2018, 2020). How can observing PFAS-induced shifts in serum lipids be linked to metabolic disease progression? Such shifts include modifications in high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triacylglycerol (TAG) levels. These blood lipids are sourced from hepatic lipids such as cholesterol and triacylglycerols and play an important role in lipid homeostasis, energy generation, and metabolic-related disease (Bhargava et al., 2022). The alterations in lipid metabolism are of particular concern because they have been associated with a spectrum of cardiovascular diseases (CVDs) and metabolic disorders, such as dyslipidemia, type 2 diabetes (T2D), and metabolic dysfunction-associated steatotic liver disease (MASLD) (Averina et al., 2021; Duan et al., 2022; Gui et al., 2023; Rinella et al., 2023; Roth & Petriello, 2022; Sen et al., 2022; Xu et al., 2023). Thus, there is a need to extensively investigate single lipid alterations driven by legacy and emerging PFAS and their roles in metabolic disease. Elucidating the relationships between PFAS exposure, lipid metabolism, and ensuing health effects is essential for identifying PFAS hazards and performing risk assessments.

Previous studies have focused primarily on conventional lipid readout parameters from clinical blood analyses, such as total cholesterol (TC), LDL-C, HDL-C, and total TAG levels, to describe PFAS-associated conditions such as dyslipidemia, i.e., a lipid imbalance driven by HDL-C versus LDL-C levels often concurrent with overall elevated TC levels (Averina et al., 2021; EFSA CONTAM Panel, 2020; Liu et al., 2023; Steenland et al., 2009). The epidemiological evidence from cross-sectional and longitudinal studies underscores a complex panorama of non-associations or associations between distinct PFAS exposures and changes in TC, LDL-C, and TAG blood levels (Dunder et al., 2022; EFSA CONTAM Panel, 2018, 2020; Li et al., 2020).

Most related studies have focused on the effects of individual legacy PFAS in human and environmental samples (mainly perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS)). We investigated the impacts of seven legacy PFAS (perfluorobutanoic acid (PFBA), perfluorodecanoic acid (PFDA), perfluorohexanoic acid (PFHxA), PFOA, perfluorohexanesulfonic acid (PFHxS), perfluorononanoic acid (PFNA), PFOS, and three novel substitutes PFAS (3 H-perfluoro-3-[(3-methoxypropoxy) propanoic acid] (PMPP/Adona), ammonium perfluoro-(2-methyl-3-oxahexanoate) (HFPO-DA/GenX), and perfluorobutanesulfonic acid (PFBS)) on lipid metabolism in vitro, within human hepatocyte-like cells (HepaRG). As the central organ for synthesizing a plethora of lipids (like cholesterol and TAG), the liver is an important site for investigating lipid metabolism changes (Cockcroft, 2021).

PFOS and PFOA have been restricted based on the persistent organic pollutant (POP) regulation (European Commission, 2019, 2020). However, novel fluorinated compounds and materials with limited toxicity data are increasingly available on the market.

The main objective of this research was to use an untargeted lipidomics approach to study lipid alterations at the level of individual lipid species and lipid classes within human HepaRG cells in response to PFAS exposure.

2. Materials and methods

2.1. Chemicals

Perfluorooctanoic acid (PFOA) (purity of 95 %), perfluorooctanesulfonic acid (PFOS) (purity ≥ 98 %), perfluorohexanoic acid (PFHxA) (purity ≥ 97 %), perfluorohexanesulfonic acid (PFHxS) (purity ≥ 98 %), perfluorobutanoic acid (PFBA) (purity ≥ 99 %), perfluorobutanesulfonic acid (PFBS) (purity ≥ 97 %), perfluorononanoic acid (PFNA) (purity ≥ 97 %), perfluorodecanoic acid (PFDA) (purity ≥ 98 %) and ammonium perfluoro-(2-methyl-3-oxahexanoate) (HFPO-DA/GenX) (purity 99 %) were obtained from Apollo Scientific (Cheshire, UK). 3 H-perfluoro-3-[(3-methoxypropoxy) propanoic acid] (PMPP/Adona) (purity > 98 %) was obtained from Campro Scientific (Berlin, Germany). Milli-Q water (Waters-Millipore Corporation, Milford, MA, USA), acetonitrile (ACN) of HPLC grade, chloroform, methanol (MeOH), and 2-propanol (IPA) were all purchased from JT Baker Chemical (Philipsburg, NJ, USA). Formic acid (purity ≥ 98 %) and ammonium formate salt (purity, 99 %) were obtained from Fluka (Steinheim, Germany). Standards containing the main lipid classes were acquired from Avanti Polar Lipids (Alabaster, AL, USA) (Table S1).

2.2. Cell culture

HepaRG hepatocarcinoma cells were acquired from Biopredic International (Saint Gregoire, France). Differentiated HepaRG cells are a mixture of hepatocyte-like and cholangiocyte-like cells (Lambert et al., 2009). The cultivation method for the HepaRG cells was previously described in detail (Lichtenstein et al., 2020). Briefly, cells were seeded in 6-well plates at a density of 200000 cells/well and were cultured in William's Medium E supplemented with 2 mM glutamine (PAN-Biotech, Aidenbach, Germany), 10 % fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany), 100 U/mL penicillin and 100 μg/mL streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany), and 5×10^{-5} M hydrocortisone hemisuccinate (Sigma—Aldrich, St. Louis, USA) at 37 °C in a humidified atmosphere with 5 % CO_2. The medium was changed every 2 days. HepaRG cells were differentiated by adding 1.7 % dimethyl sulfoxide (DMSO) to the medium after 14 days of proliferation and cultivated further in this differentiation medium for another 14 days. After four weeks of preparation (2 weeks of proliferation + 2 weeks of differentiation), the treatment medium was replaced with the same composition as the differentiation medium but containing only 2 % FBS and 0.5 % DMSO. After 48 hours, the cells were incubated with various concentrations of PFAS diluted in DMSO for 72 hours, and the final DMSO concentration was 0.5 % in the treatment medium for both control and exposed cells. Based on previous results on cytotoxicity (Sadrabadi et al., 2024), three non-cytotoxic concentrations were evaluated for each tested compound: PMPP/Adona (100, 250, 1000 μM); HFPO/GenX (100, 1000, 5000 μM); PFBA (100, 1000, 5000 μM); PFBS (100, 1000, 2500 μM); PFDA (10, 25, 100 μM); PFHxA (100, 1000, 5000 μM); PFHxS (100, 250, 500 μM); PFNA (10, 25, 100 μM); PFOA (25, 100, 250 μM); PFOS (10, 25, 100 μM). PFNA 25 μM sample was not measured in positive mode due to a technical error. Four independent replicates per concentration were studied.

2.3. Lipidomics

Lipids were extracted from the HepaRG cells by adding internal standards (Splash mix, Advanti) (Table S1) and ice-cold extraction

solvent (MeOH: water: chloroform (2:3:2, v/v/v)), followed by vigorous shaking using a Precellys 24 Dual device (Bertin Technologies, Aix-en-Provence, France) (6500 rpm for 2 cycles of 10 s with a 15 s break between the cycles. Subsequently, the samples were placed on ice for 10 minutes to allow partitioning and protein precipitation. Then, the samples were centrifuged (12000 × g for 10 minutes at 4 °C) to precipitate the cell material and separate the polar from the apolar fractions. The bottom layer (100 μL), containing the apolar metabolites (e.g., lipids), was transferred to a glass vial, dried using nitrogen gas, and reconstituted in ACN: IPA: Milli-Q water (5:4:1, v/v/v). An aliquot (10 μL) was taken to prepare a pooled sample for quality control (QC) analysis. The samples were stored at −80 °C before lipidomics analysis.

Liquid chromatography (LC) combined with mass spectrometry (MS) was used for comprehensive lipid profiling. A 1290 LC system (Agilent, Palo Alto, CA) coupled to a quadrupole time-of-flight MS (Q-TOF, Compact, Bruker Daltonic, Bremen, Germany) was used. The Q-TOF was run in positive and negative modes using an electrospray ionization (ESI) source. The lipids were separated by a Linetex EVO C18 LC column (2.1 mm × 100 mm, 2.6 μM) (Phenomenex, USA). The mobile phases were solvent A (Milli-Q water: ACN, 40:60 %, v/v) and solvent B (ACN: IPA, 10:90 %, v/v). Ammonium formate (10 mM) and 0.1 % formic acid were added to mobile phases A and B. The LC gradient was as follows: 0.3 min 15 % B, 2 min 30 % B, 3 min 48 % B, 15 min 82 % B, 16 min 99 %, 20 min 99 %, 21 min 15 % B, and 23 min 15 % B. The flow rate was 0.3 mL min^{-1}, the column temperature was 45°C, and the injection volume was 5 μL.

The MS data were run in auto MS/MS acquisition mode with 5 spectra/sec. The settings for the MS were as follows: capillary voltage at ± 500*V* and ± 400*V* in positive and negative modes, respectively; end plate pressure of ± 500*V*; nebulizer gas (N_2) pressure of 4 bar; drying gas flow rate of 6 L min^{-1}; and drying gas temperature of 200 °C and 250 °C in positive and negative modes, respectively. The scan range was *m/z* from 60 to 2000 for the positive mode and 50–2000 for the negative mode. For the calibration of the MS data, the sodium format was used for internal mass calibration and the pooled samples. Quality control (QC) samples were analyzed after every 9th sample. For quality control, we randomized the samples for LC-MS analysis. After data filtration and normalization, the mean relative standard deviation (RSD) of the QC samples in the positive and negative modes were 24 % and 34 %, respectively.

2.4. Data processing and statistical analysis

After internal mass calibration, the LC-Q-TOF data were converted into the '.mzML' datafile format using the Bruker data analysis package (Bruker Daltonic, Bremen, Germany). Subsequently, the data were imported into MS-DIAL 4.92 for peak alignment and lipid annotation (Tsugawa et al., 2015). The settings for lipid annotation are shown in Table S2. Lipid features were annotated based on the retention time similarity score, accurate mass, isotope pattern, and MS/MS spectra. The total score of the annotated lipids was set at 80 %. The annotated lipids were further processed with NOREVA 2.0 (Fu et al., 2022) for data filtering, imputation of missing values, QC sample correction, and normalization. Missing values were imputed using the mean value of the 5 neighbors in the datasets via the k-nearest neighbors algorithm (k-NN). QC sample drift was corrected using local polynomial fits, and peak intensities were normalized using EigenMS (Karpievitch et al., 2014). The normalized data were then imported into Metaboanalyst 5.0 for statistical analysis (Chong et al., 2019). The normalized data were scaled using Pareto scaling for principal component analysis (PCA). Statistical analysis was based on one-way ANOVA ($p < 0.05$; Fisher's post hoc test and false discovery rate (FDR) correction). Concentration-response analysis was performed using four independent biological samples, and enrichment analysis was conducted using a web-based lipid pathway enrichment analysis (LIPEA) (BIOTEC, Dresden, Germany).

3. Results

The differentiated HepaRG cells were exposed to ten PFAS at various concentrations. The cytotoxicity potentials of these PFAS were screened at various concentrations, as described in our previous work (Sadrabadi et al., 2024). Based on the aforementioned study, three concentrations of each PFAS, defined as non-cytotoxic, were selected for exposure prior to lipidomic analysis. 166 annotated lipid features were found in HepaRG cells, covering a broad range of lipid subclasses. These subclasses, in turn, cover a wide range of lipids, including glycerophospholipids, sphingolipids, glycerolipids, fatty acyls, fatty amides, sterols, and phenols (Table 1). The largest number of annotated lipid species belonged to glycerophospholipids (n=74), followed by glycerolipids (n=49) (see supplementary material for a full overview of annotated features in positive and negative mode).

Hierarchical cluster analysis (HCA) of samples and lipids revealed that some PFAS clustered together, indicating similar effects on individual lipid species (Fig. 1). PFOS (10, 25, and 100 μM), PFOA (25, and 100 μM), and PFDA (25 μM) formed one cluster, whereas PFDA (10, and 100 μM), PFNA (10, and 25 μM), PFOA (250 μM) and Adona (100 μM) clustered together. PFBS (1000 and 2500 μM) and PFHxA (1000 μM) also clustered together. The highest concentrations tested (PFBA 5000 μM, HFPO-DA/GenX 5000 μM, and PFHxA 5000 μM) also formed a distinct cluster, deviating from the lower tested concentrations.

Based on this cluster analysis, there is no clear distinction of PFAS chain length; however, PFOA and PFOS with the C8 chain length clustered together, except for 250 μM PFOA.

Our findings showed that exposing HepaRG cells to specific concentrations of PFOS (10 and 25 μM) and PFOA (25 and 100 μM) led to increased levels of several lipid species (ceramide (Cer), diacylglycerol (DAG), N-acyl ethanolamine (NAE), phospholipid (PC), and triacylglycerol (TAG)). However, exposure to PMPP/Adona (1000 μM), HFPO-DA/GenX (100 and 5000 μM), PFBA (100 and 5000 μM), PFHxA (100 and 5000 μM), or PFHxS (500 μM) resulted in decreased levels of these lipids. Interestingly, PFDA 25 μM showed a similar effect to PFOS and PFOA, although it was not statistically significant in many cases. Notably, Adona (100 and 250 μM), HFPO-DA/GenX (100 and 1000 μM), PFBA (1000 μM), PFBS (100, 1000, and 2500 μM), PFDA (10 and 100 μM), PFHxS (100, 250, and 500 μM), and PFNA (10, 25, 100 μM) had mixed effects on different lipid species, as illustrated in Fig. 2. For example, PFBA (100 and 1000 μM) increased fatty acid (FA), lysophosphatidylcholine (LPC), and lyso-phosphatidylethanolamine (LPE) levels but decreased TAG levels.

In HepaRG cells exposed to PFOA (25, 100, and 250 μM) and PFOS (10, 25, and 100 μM), saturated fatty acids (SFAs) such as palmitic acid (FA 16.0), margaric acid (FA 17.0) and myristic acid (FA 14.0) were decreased (Fig. 2). Moreover, HFPO-DA/GenX (100 μM), PFBA (5000 μM), and PFHxA (5000 μM) led to a reduction in the levels of FA, LPC, and LPE. PFOS (250 μM), PFHxA (1000 and 5000 μM), PFDA (10 and 25 μM), PFBS (100 μM), PFBA (5000 μM), and HFPO-DA/GenX (100 and 5000 μM), and PMPP/Adona (100 and 1000 μM) caused a decrease in LPC (16.0) levels. The highest HFPO-DA/GenX, PFBA, and PFHxA concentrations consistently decreased most lipid levels, likely due to early signs of cytotoxic response, and were therefore not investigated further.

Concentration-response assessments of single PFAS and individual lipid species revealed distinct patterns of lipid alterations and indicated that some lipid changes were concentration-dependent. (Fig. 3). For instance, after exposure to PFDA, HepaRG cells showed a concentration-dependent increase in Cer (21:2; O2.16:2) and Cer (29:3; O2.18:5). Similarly, PFOS-exposed cells had increased DAG (18.1_22.6) and ether-PC (PCO) (17.0_16.1) levels. In contrast, a concentration-dependent decrease in DAG (40.2) was observed in HepaRG cells treated with PFHxA, while PFHxS caused a reduction in PCO (11.0_22.3). PFBA and PFOA exposure also decreased FA (17.0) and FA (16.0) levels.

We performed pathway enrichment analyses based on our

Table 1
Overview of annotated lipid classes showing downregulated lipids (**blue**), upregulated lipids (**red**), and both upregulated and downregulated lipids (**yellow**). Each lipid subclass is denoted with an up (left number) or down (right number) tag, signifying an increase or decrease, respectively. The notation 'N' represents each subclass's total number of annotated lipid features.

(Up/Down) Significantly altered lipid levels	N	Adona			GenX			PFBA			PFBS			PFDA			PFHxA			PFHxS			PFNA			PFOA			PFOS		
		Adona 100	Adona 250	Adona 1000	GenX 100	GenX 1000	GenX 5000	PFBA 100	PFBA 1000	PFBA 5000	PFBS 100	PFBS 1000	PFBS 2500	PFDA 10	PFDA 25	PFDA 100	PFHxA 100	PFHxA 1000	PFHxA 5000	PFHxS 100	PFHxS 250	PFHxS 500	PFNA 10	PFNA 25	PFNA 100	PFOA 25	PFOA 100	PFOA 250	PFOS 10	PFOS 25	PFOS 100
Glycerophospholipids																															
Bismonoacylglycerophosphate (BMP)	2	0/0	0/0	0/0	0/0	0/0	0/2	0/1	0/0	0/2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/2	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Lysophosphatidylcholines (LPC)	10	0/1	0/1	0/2	0/2	0/1	0/10	0/0	1/0	0/7	2/3	0/1	0/0	0/5	0/2	1/0	0/0	0/2	0/8	0/0	0/1	0/0	0/1	0/1	0/0	0/1	0/0	0/2	0/0	0/0	0/1
Phosphatidylcholine (PC)	40	6/1	3/3	7/6	2/1	2/0	2/7	8/0	3/2	1/9	5/0	4/1	3/4	5/4	4/1	6/2	3/3	6/5	2/10	4/0	3/4	6/6	3/1	2/0	6/1	7/0	4/0	2/0	2/1	6/0	7/0
Phosphatidylethanolamine (PE)	12	2/0	1/0	2/1	1/0	0/0	0/3	3/0	0/0	0/2	3/0	1/0	0/0	2/1	3/0	4/0	3/1	2/0	1/6	0/0	0/1	0/1	3/1	0/0	3/0	0/0	1/0	0/0	0/1	3/0	4/0
Lysophosphatidylserine (LPS)	1	0/0	0/0	0/1	0/0	0/0	0/1	0/0	0/0	0/1	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/1	0/1	0/0	0/1	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0
Phosphatidylglycerol (PG)	2	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/2	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/1	0/0	0/0	0/0	0/0
Lysophosphatidylethanolamine (LPE)	7	0/0	0/0	0/4	0/0	0/2	0/5	1/0	0/0	0/4	0/0	0/0	0/2	0/5	0/3	0/0	0/0	0/2	0/5	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/1	0/0	0/0	0/1
Sphingolipids																															
Ceramides (Cer)	13	0/0	0/1	2/0	1/0	0/0	1/1	1/0	0/1	1/2	0/0	0/1	0/0	1/1	0/2	0/0	1/0	1/0	3/1	0/0	2/0	1/1	1/0	0/0	1/0	3/0	3/0	0/0	4/0	4/0	4/0
Sphingomyelin (SM)	4	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Sulfonolipid (SL)	3	0/0	0/0	0/2	0/0	0/0	0/2	0/0	0/0	0/2	0/0	0/0	0/0	0/2	1/0	0/2	0/1	0/2	0/2	0/0	1/0	0/2	0/1	0/0	1/1	2/0	2/0	0/0	1/0	1/0	1/0
Glycerolipids																															
Monoacylglycerol (MG)	1	0/0	0/0	0/0	0/0	0/0	0/0	0/1	0/0	0/1	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Diacylglycerol (DAG)	29	1/3	1/2	1/8	3/1	1/1	0/11	1/1	1/3	2/9	4/1	4/2	3/2	0/7	2/5	1/5	1/3	2/5	0/11	2/1	2/2	2/4	0/2	0/0	1/2	6/0	6/2	2/2	5/0	4/0	3/1
Triacylglycerols (TAG)	19	3/3	1/1	0/13	0/9	2/0	0/22	0/12	0/5	0/18	0/10	0/11	3/8	2/2	7/2	0/4	0/10	2/11	0/23	2/1	0/13	0/16	1/0	0/0	1/3	15/0	14/0	1/5	19/0	8/0	5/1
Fatty acyls																															
(FA)	16	0/1	0/0	0/1	0/1	0/0	0/14	0/0	0/0	0/18	0/1	0/0	0/0	0/2	1/1	1/0	0/0	0/1	0/19	0/0	0/0	0/0	0/1	0/5	0/0	0/5	0/3	0/2	0/2	0/2	0/3
Fatty amides																															
N-acyl ethanolamines (NAE)	3	0/1	0/0	0/0	0/1	0/0	0/2	0/0	1/0	0/2	0/0	0/0	0/0	1/1	1/0	1/1	0/0	0/1	0/0	0/0	0/0	0/1	1/1	0/0	0/1	1/0	1/0	0/1	1/1	1/0	1/0
Sterols																															
Sterol sulfate (ST)	2	0/0	0/0	0/2	0/0	0/0	0/2	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/2	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Prenols																															
Vitamin A ester (VAE)	2	0/0	0/0	0/0	0/2	0/0	0/0	0/2	0/0	0/2	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/2	0/2	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Total of affected lipid	166																														

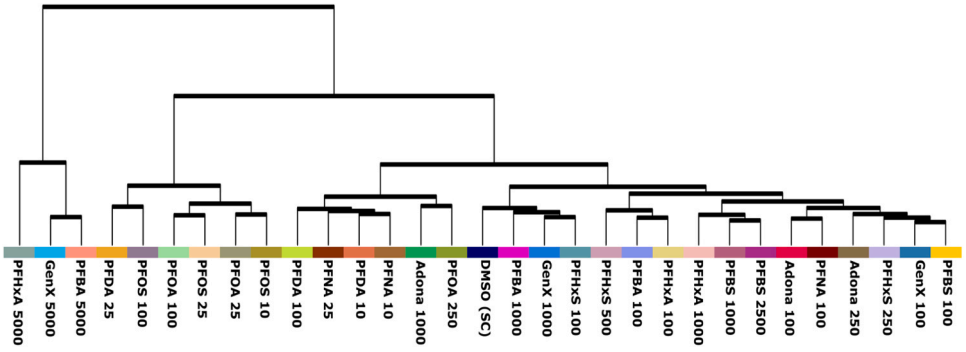

Fig. 1. Hierarchical cluster analysis of ten individual PFAS at different concentrations based on normalized lipid features in HepaRG cells. Annotated lipids from both positive and negative ionization modes.

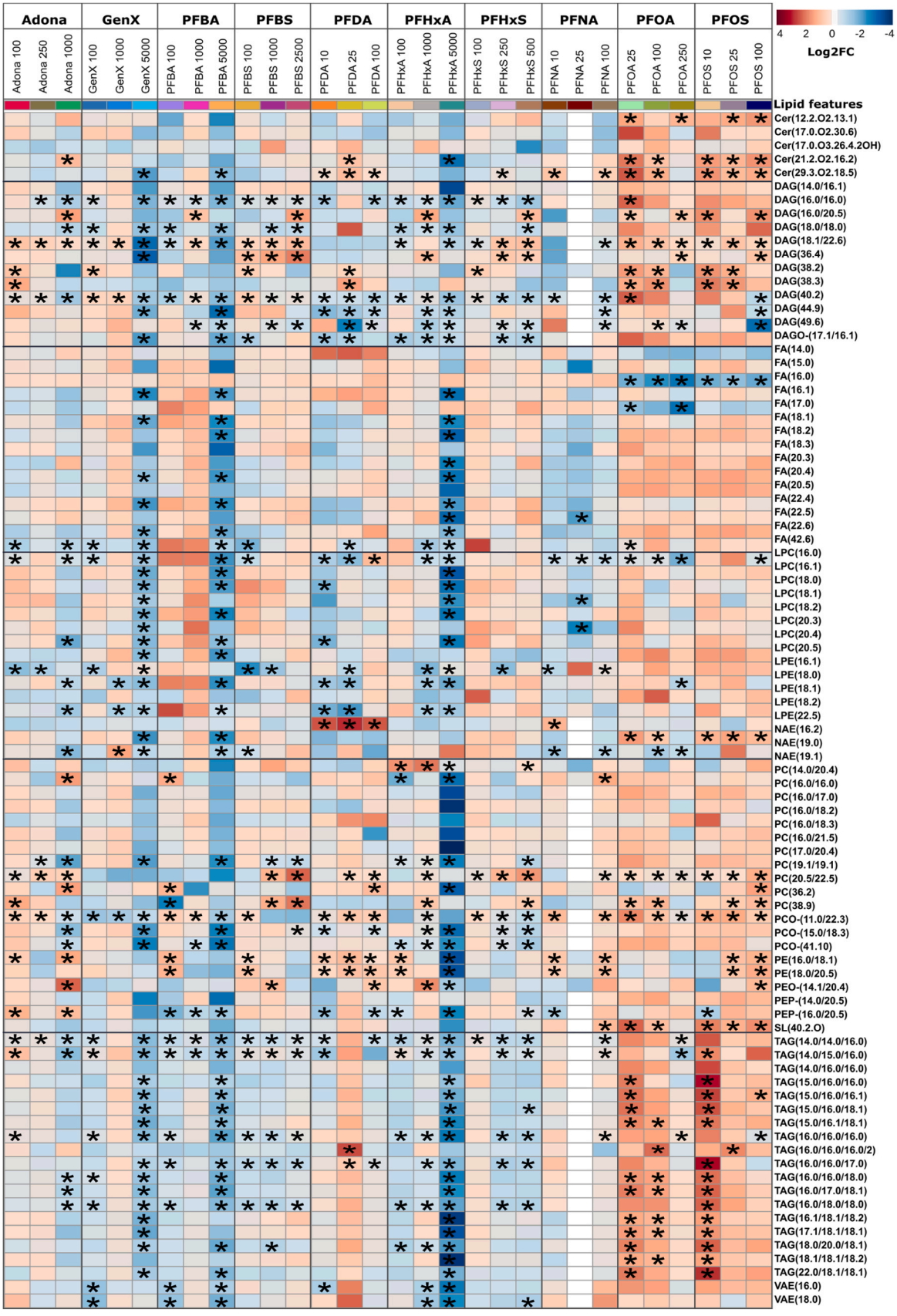

Fig. 2. Heatmap of normalized response (mean of four biological replicates) of the topmost statistically significant lipid features in positive and negative ionization mode. Results for the PFNA 25 μM sample are missing for the positive ionization mode (white fields) as these samples were not measured due to a technical error. Statistically significant (* $p < 0.05$) differences between PFAS and the solvent control (DMSO) are indicated by an asterisk (*), and the scale is shown as a Log2 fold change. **Blue** indicates a decrease, and **red** indicates an increase.

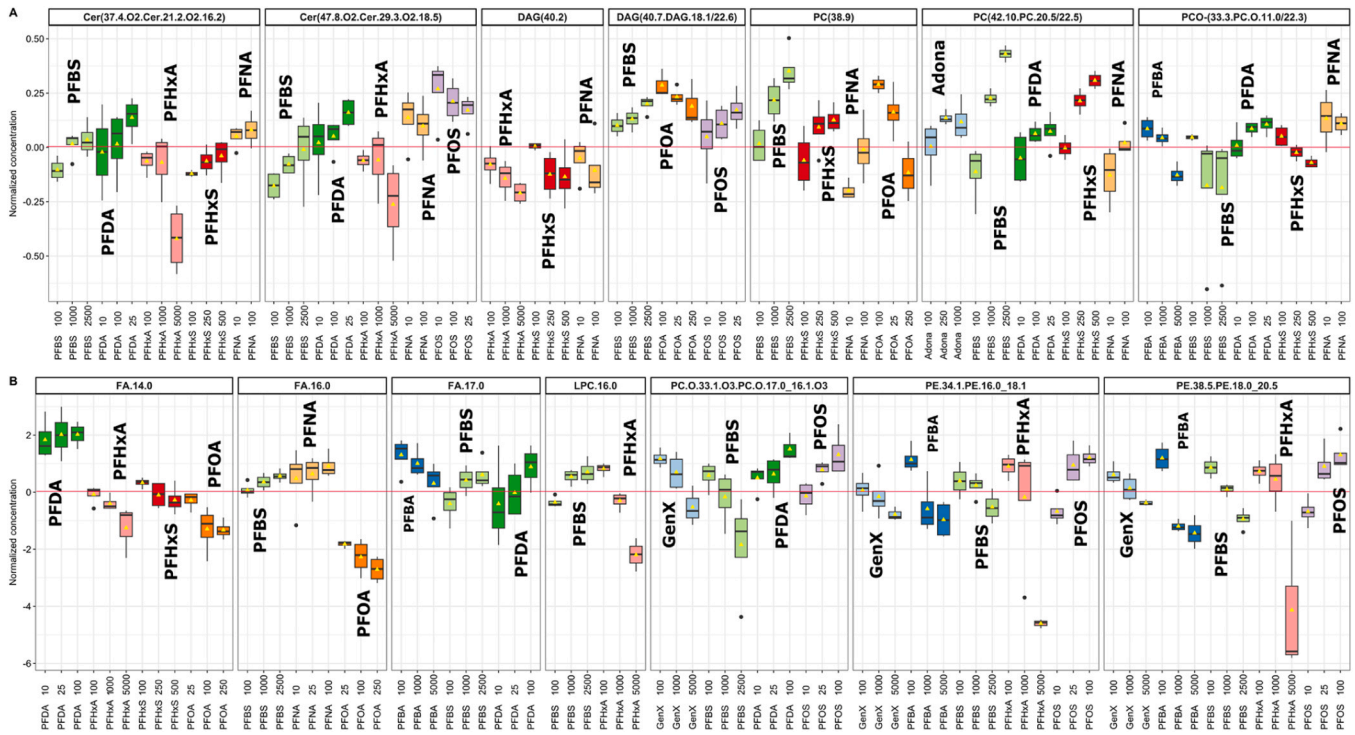

Fig. 3. PFAS concentration-dependent relationships for selected individual lipids. The differences (one-way ANOVA) of normalized data of individual PFAS compared to the control are shown. A) Lipid features detected in positive ionization mode. B) Lipid features detected in negative ionization mode. Normalized concentration is based on the normalized response compared to the solvent control (DMSO), which was set to zero (red horizontal line).

statistically significant generated lipid features to determine lipid involvement in critical biological processes, including disease. Among the twelve enriched pathways, the five top pathways include "Glycerophospholipid metabolism", "Sphingolipid signaling", "Insulin resistance", "Choline metabolism in cancer", and "AGE-RAGE signaling pathway in diabetic complications", as shown in Table 2.

Table 2
Pathways significantly enriched in lipids by PFAS-mediated effects.

Pathway name	Pathway lipids	Raw p-value	Benjamini corrected p-value	Lipid class
Glycerophospholipid metabolism	26	0.0000	0.0000	LPC, PE, DAG, LPE, PC
Sphingolipid signaling pathway	9	0.0002	0.0046	DAG, Cer
Insulin resistance	4	0.0002	0.0046	TAG, DAG, Cer
Choline metabolism in cancer	5	0.0004	0.0085	PC, LPC, DAG
AGE-RAGE signaling pathway in diabetic complications	2	0.0013	0.0211	DAG, Cer
Retrograde endocannabinoid signaling	8	0.0022	0.0295	PE, DAG, PC
Neurotrophin signaling pathway	3	0.0039	0.0309	DAG, Cer
Long-term depression	3	0.0039	0.0309	DAG
Adipocytokine signaling pathway	3	0.0039	0.0309	DAG
Kaposi's sarcoma-associated herpesvirus infection	3	0.0039	0.0309	DAG, PE
Ferroptosis	11	0.0061	0.0441	PE
Necroptosis	4	0.0077	0.0504	Cer

4. Discussion

4.1. Lipid alterations

In this study, our findings revealed various alterations in the lipid profiles of HepaRG cells after exposure to different single PFAS. The results demonstrate the potential of PFAS to disrupt lipid homeostasis and metabolism in hepatic cells, with distinct effects observed for each PFAS tested. Specifically, PFOA and PFOS caused a notable increase in TAG and DAG species, along with elevated levels of PC and PE (Fig. 2, **S1**). These alterations in the hepatic lipid profile appear to be mediated primarily by PPARα and, to a lesser extent, PPARγ, CAR, and PXR. This phenomenon prompts questions regarding possible relationships with health effects, such as metabolic-related diseases, T2D, CVDs, and MASLD.

Numerous studies have examined PFAS exposure and its effects on lipid metabolism using different models in HepaRG cells (Behr et al., 2020; Franco et al., 2020; Louisse et al., 2020; Sadrabadi et al., 2024), in rodents (Das et al., 2017; Rosen et al., 2010; Wu et al., 2023) and in several human cohort studies (Averina et al., 2021; Fenton et al., 2021; Jain & Ducatman, 2019; Steenland et al., 2009) and reported PFAS to alter lipid metabolism; for example, the elevation of TAG levels (Dalla Zuanna et al., 2021; Dunder et al., 2022; Jain & Ducatman, 2019; Li et al., 2020; Zare Jeddi et al., 2022). However, the landscape of studies on these effects is multifaceted, with epidemiological evidence showing no associations or positive or negative associations between PFAS exposure and serum lipid levels (Dunder et al., 2023; Schillemans et al., 2023; Zheng et al., 2023). Therefore, this study aimed to explore the impact of PFAS on lipid metabolism in HepaRG cells, focusing on lipid composition, known as the lipidome.

Previous research suggests that PFAS effects are primarily PPARα-mediated, with contributions from other nuclear receptors (NRs) such as PPARγ, CAR, and PXR (Attema et al., 2022; Bjork et al., 2011; Rosen et al., 2017; Vanden Heuvel et al., 2006). These NRs play crucial roles in regulating lipid metabolism, with PPARγ regulating lipogenesis and the

import of free fatty acids (Skat-Rørdam et al., 2019), while CAR and PXR regulate energy metabolism and promote hepatic lipid accumulation (Marmugi et al., 2016; Nakamura et al., 2007; Yamamoto et al., 2003). PPARα regulates fatty acid oxidation and gene expression in TAG metabolism (Pawlak et al., 2015). It is the most commonly reported NR activated by PFAS, including but not limited to PFOA, PFOS, PFHxA, PFHxS, PFBA, and PMPP/Adona (Kirk et al., 2021; Maeda et al., 2024; Shi et al., 2024). PFOA and PFOS have also been shown to upregulate TAG-associated gene expression (ATSDR, 2021; Louisse et al., 2020). TAG acts as a reservoir for FAs, releasing them to provide energy under strict instruction when needed. TAG consists of fatty acid chains of varying lengths, which can be either saturated (SFAs) or unsaturated, such as polyunsaturated FAs (PUFAs). Most of these FAs are known activators of PPARα and essential components of phospholipids in the cell membrane, playing a crucial role in maintaining membrane fluidity and integrity (Leekumjorn et al., 2009).

PUFAs such as arachidonic acid (FA, 20.4) serve as a substrate for the CYP4A enzyme, crucial in FA metabolism and homeostasis (Jarrar & Lee, 2019; Zhang & Klaassen, 2013). Additionally, the activation of PPARα has been found to consistently increase the expression of CYP4A11, promoting fatty acid oxidation (Bjork et al., 2011). Alteration in the metabolism of SFAs and PUFAs can disrupt the biosynthesis of TAG and other lipids, negatively impacting the biological system (Perry et al., 2014; Postic & Girard, 2008). For example, TAG accumulation can lead to hepatic steatosis and MASLD (Alves-Bezerra & Cohen, 2017; Browning et al., 2011; Das et al., 2017; Jiang et al., 2015; Semova & Biddinger, 2021; Sen et al., 2022; Yamaguchi et al., 2007).

4.1.1. PFOS, PFOA, and PFDA induce lipid accumulation

The lipid profile of HepaRG cells exposed to PFOA (25 and 100 μM), PFOS (10, 25, 100 μM), and PFDA (25 μM) exhibited significant differences compared to those exposed to other tested PFAS. PFOS and PFOA notably elevated the levels of most lipid species, except for SFAs (FA with 14.0, 16.0, and 17.0 carbon chains), which were decreased. Interestingly, these two PFAS increased TAG species containing one or two SFAs with 14.0, 16.0, or 17.0 fatty acyl chains and PUFAs. Examples are DAG (16.0/16.0), DAG/ether-PE (PEP) (16.0/20.5), TAG (16.0/16.0/16.0), TAG (14.0/14.0/16.0), TAG (14.0/16.0/16.0), and TAG (16.0/16.0/17.0), and TAG species containing PUFAs, including TAG (16.1/18.1/18.2), TAG (17.1/18.1/18.2), and TAG (18.1/18.1/18.2) (Fig. 2). This finding suggests that in cells exposed to PFOA- and PFOS, SFAs and PUFAs were used in the esterification of TAG (Clarke, 2001; Zhang et al., 2016). Similarly, Franco and colleagues reported increased DAG and TAG species containing PUFAs in HepaRG exposed to PFOA (Franco et al., 2020), suggesting a PPARα-dependent mechanism, as PFOS and PFOA exposures in HepaRG cells are associated with increased fat storage (TAGs) and potentially decreased fatty acid oxidation (Das et al., 2017; Lindquist et al., 2017). PFDA, which has been found to elevate TAG levels in rodents (Van Rafelghem et al., 1988; Wang et al., 2023), exhibited a similar effect in our exposed HepaRG cells at 25 μM PFDA. Moreover, the observed increase in TAG levels may also result from the activities of PPARγ, PXR, and CAR, which may occur as a secondary effect of PPARα activation or suppression (Attema et al., 2022; Attema et al., 2024; Bjork et al., 2011; Rosen et al., 2017; Vanden Heuvel et al., 2006).

Elevated TAG levels in cells exposed to PFOS, PFOA, and PFDA (25 μM) suggest lipid accumulation (Das et al., 2017; Rosen et al., 2010; Yang et al., 2023), likely due to increased fatty acid load, reduced fatty acid oxidation, and an increase in the synthesis of TAG (Angrish et al., 2016). In these exposure conditions, fatty acid oxidation seems to be inhibited, shifting from oxidation to esterification to form glycerolipids and phospholipids. Moreover, lipid metabolism in the liver is balanced between lipid synthesis and energy generation, and any disruption of this balance can either cause lipid accumulation or oxidation (Fang et al., 2019). The elevation of PUFAs, a decrease in SFAs, and the increased incorporation of FAs into DAG, TAG, and phospholipids may cause an imbalance in the PUFA and SFA levels. These alterations can disrupt energy homeostasis and lipid equilibrium, altering lipid composition and intermediates and potentially generating toxic lipid species (Vendruscolo, 2022). Excess PUFAs can increase lipid peroxidation, alter cell membrane structure and dynamics, and trigger cell death (Gaschler & Stockwell, 2017). Further, a decrease in SFAs may reduce the formation of PUFAs, which are key in preventing metabolic disorders (Beauchamp et al., 2009; Rioux et al., 2008; Shramko et al., 2020). Also, alterations in the levels of PC and PE can disrupt calcium homeostasis and induce endoplasmic reticulum stress (ER stress) (Moncan et al., 2021; Patel & Witt, 2017).

4.1.2. Impacts of PFHxS, PFHxA, PFBS, PFBA, and HFPO-DA/GenX on lipid profile

PFHxS, PFHxA, PFDA, PFBS, PFBA, and HFPO-DA/GenX exhibited a non-concentration-dependent increase of PUFAs and phospholipids. HFPO-DA/GenX has been found to reduce TAG levels in rats (Conley et al., 2021), and at 2500 μM concentrations increased the total level of TAG in HepaRG cells using adipored assay (Sadrabadi et al., 2024); our finding shows a decrease in TAG levels at HFPO-DA/GenX (100 μM).

PFHxS exposure has been linked to lipid accumulation in zebrafish embryos (Ulhaq & Tse, 2024) and rodents (Bijland et al., 2011; Das et al., 2017; Pfohl et al., 2020), as well as decreased serum TAG levels in rats (ATSDR, 2021; Bijland et al., 2011). Our study observed reduced TAG species levels in HepaRG cells exposed to PFHxS, independent of concentration. Conversely, PFNA exposure studies have reported decreased serum TAG levels in mice (Wang et al., 2015), elevated hepatic TAG levels in PPARα-null mice (Das et al., 2017) and HepaRG cells (Louisse et al., 2020). In contrast, our results showed minimal impact of PFNA on TAG levels. PFBS and PFHxA exposure were associated with reduced TAG levels in rodents (Bijland et al., 2011; Klaunig et al., 2015), which is consistent with our findings. In contrast, Sadrabadi et al. (2024) reported increased TAG levels in HepaRG cells exposed to PMPP/Adona (1000 μM), whereas we found reduced TAG levels at the same concentration. It is worth noting that the adipored assay measures total TAG levels without distinguishing TAG species, whereas the lipidomics approach discriminates among TAG species, making comparing findings from these two methods challenging.

The observed decrease in TAG and, to a lesser extent, DAG lipid species in cells treated with 100 μM HFPO-DA /GenX, PFBS, PFBA, PFHxA, and PFHxS suggests a promotion of lipid oxidation by these PFAS, diverting away from TAG biosynthesis. Consequently, the increased levels of FA could promote the generation of other lipid species (De Carvalho & Caramujo, 2018). For instance, exposure to PFHxS (250 μM) and PFBA (100 μM) led to an increase in SFAs (FA with 15.0, 16.0, and 17.0 carbon chains) with a significant reduction in TAG species containing these FAs, such as TAG (14.0/15.0/16.0), TAG (16.0/16.0/16.0) and TAG (16.0/16.0/17.0). These outcomes may indicate lipid remodeling and disruption in energy homeostasis (Syed-Abdul, 2023).

4.2. Pathway analysis

Our enrichment analysis uncovered pathways potentially linked to cardiovascular effects and diabetes. For example, AGE-RAGE signaling is implicated in the development and progression of diabetic complications, including cardiovascular disease. (Kahn et al., 2006). An example of the relationships between DAG, TAG, and Cer levels and insulin resistance according to pathway enrichment analyses is discussed below and illustrated in Fig. 4. Specifically, the PI3K/AKT pathway is involved in insulin resistance, reducing glycogen production and de novo lipogenesis and activating gluconeogenic genes. It is important to note that the relationship between these pathways and disease outcomes is complex and can vary depending on individual factors and disease states. Therefore, further research is needed to elucidate these connections fully.

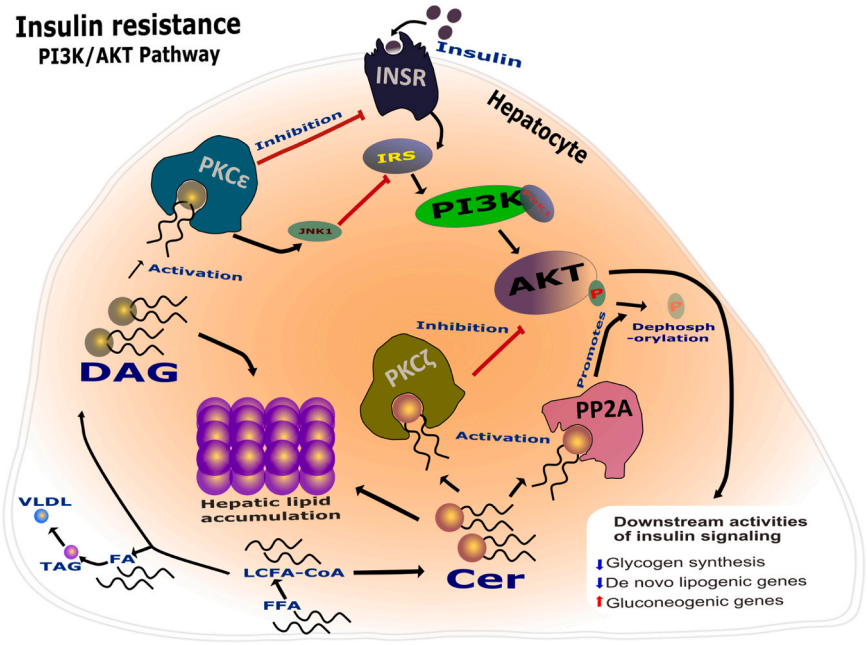

Fig. 4. Role of diacylglycerol (DAG) and ceramide (Cer) in insulin resistance. DAG inhibits the stimulation of insulin receptors (INSRs) upon insulin binding by activating protein kinase C epsilon (PKCε). Cer activates protein kinase C zeta (PKCζ) and protein phosphatase 2 A (PP2A), which block serine-threonine kinase (AKT) activation and promote AKT dephosphorylation. Long-chain fatty acids can be used as building blocks for the biosynthesis of organisms' triacylglycerols (TAGs) and ceramides. Dysregulated AKT in insulin resistance promotes gluconeogenesis activation, decreasing de novo lipogenesis (DNL) and glycogen synthesis.

DAG serves as a precursor for TAG biosynthesis and a critical signaling molecule. DAG inhibits insulin signaling by activating protein kinase C epsilon (PKCε) (Petersen & Shulman, 2018). This activation blocks insulin receptors (INSRs). It stimulates c-Jun N-terminal kinase (JNK1), hindering the activation of insulin receptor substrate 2 (IRS-2) and thus preventing the translocation and phosphorylation of AKT2 (Lopez-Bergami et al., 2005; Yung & Giacca, 2020). AKT2 is a serine/threonine kinase in the insulin signal transduction cascade that acts as an effector molecule, and its activation is also inhibited when ceramide activates protein phosphatase 2A (PP2A) and protein kinase C zeta (PKCζ), which blocks AKT2 activity (Sokolowska & Blachnio-Zabielska, 2019). Consequently, impaired PI3K/AKT2 pathway activity in insulin resistance leads to decreased glycogen synthesis and de novo lipogenesis but increased hepatic gluconeogenesis (Petersen & Shulman, 2018).

The recent understanding of DAG and Cer in insulin resistance highlights their significance beyond energy generation and cellular membrane composition. The role of these lipids in the insulin resistance pathway suggests that elevated levels of these lipids, as observed with PFAS (PFOS and PFOA) exposure, might contribute to the development of insulin resistance. However, the extent to which these lipid alterations lead to such effects remains to be elucidated.

4.3. Relevance and limitations

It is worth noting that high PFAS levels, as used in our study, are generally not observed in the population (EFSA CONTAM Panel, 2020). Instead, humans are usually exposed to lower levels of a PFAS mixture dominated by PFOS and PFOA. The reported median serum concentrations within the European adult population were 7.7 ng/mL (=0.015 μM) for PFOS and 1.9 ng/mL (=0.005 μM) for PFOA as of 2020 (EFSA CONTAM Panel, 2020). These concentrations are considerably lower than the test concentrations in this study, i.e. (10 – 100 μM for PFOS) and PFOA (25 – 250 μM for PFOA). However, PFAS serum levels are reported in highly exposed populations from contaminated areas, and the maximum reported levels can be in the range of our test concentrations. The individual with the highest recorded PFOS levels in the C8-cohort (USA) had a concentration of PFOS reaching 35.5 μM (Steenland et al., 2009). In Ronneby (Sweden), PFOS levels in serum of 0.35 μM have been reported (Li et al., 2018), and the maximum serum levels of PFOA from the Veneto area (Italy) was 6.6 μM (Batzella et al., 2022). Notably, our results may not be relevant to human health issues, but they were used to understand the mechanism of PFAS toxicity at the molecular level.

The transportation of PFAS into hepatocytes has been investigated in various studies and depends on PFAS's affinity to hepatic proteins (Chen & Guo, 2009), as illustrated by PFOS entering the hepatic cell (Fragki et al., 2023). PFOS has a higher affinity to hepatic proteins such as liver fatty acid binding protein (L-FABP) than PFNA and PFHxS (Zhang et al., 2013). Uptake of PFAS into the hepatic cell was also reported by Franco and colleagues, who observed a decrease in PFOA and PFOS in cell culture media after 7 days of exposure (Franco et al., 2020).

Hepatocyte-like cells derived from a human hepatocellular carcinoma, as used in our study (HepaRG), might have a slightly different metabolism profile than primary human hepatocytes (Gripon et al., 2002; Rogue et al., 2012; Samanez et al., 2012). Moreover, only intracellular lipids have been determined in the present study, and extracellular lipids were not analyzed due to the presence of lipids in the serum of the cell culture medium.

5. Conclusions

We investigated the impact of ten individual PFAS on the hepatocyte lipidome, showing a notable difference between legacy PFAS and their alternatives, consolidating the current literature on PFOS and PFOA, and highlighting PFDA's possibility to disrupt HepaRG lipidome similar to PFOA and PFOS, contrasting with the observed lipid alteration in cells treated with HFPO-DA /GenX, PFBS, PFBA PFHxA, and PFHxS. Our findings provide valuable insights into how single PFAS, at varying concentrations, affect lipid metabolism at the lipid subclass level. Future studies should investigate the impact of PFAS mixtures on the hepatocyte lipidome and compare them with current findings.

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CRediT authorship contribution statement

Pim E.G. Leonards: Writing – review & editing, Supervision, Software, Resources, Methodology, Formal analysis, Data curation, Conceptualization. **Timo Hamers:** Writing – review & editing, Supervision. **Lackson Kashobwe:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lars Brunken:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Faezeh Sadrabadi:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Torkjel M. Sandanger:** Writing – review & editing, Supervision. **Ana Carolina M. F. Coelho:** Writing – review & editing, Visualization, Software, Formal analysis. **Albert Braeuning:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Mattias Öberg:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Thorsten Buhrke:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2024.153862.

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