Environment International 173 (2023) 107838

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Full length article

Alteration of hepato-lipidomic homeostasis in A/J mice fed an environmentally relevant PFAS mixture

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ARTICLE INFO

Handling Editor: Adrian Covaci

Keywords:
Lipids
PFAS mixture
Mammals
Ski products
Environmental contamination
Human health

ABSTRACT

In the present study, we have investigated liver lipid homeostasis and corresponding changes in transcript and functional product levels in A/J mice exposed to environmental relevant concentration of per- and polyfluoroalkyl substances (PFAS) mixture. Mice were fed environmentally relevant concentrations of a PFAS mixture during a period of 10 weeks. The concentrations of the 8 individual PFAS in the mixture were chosen based on measured concentrations in earthworms at a Norwegian skiing area. Our data show high liver accumulation of ∑PFAS in exposed mice, which paralleled significant elevation in body weight and hepatosomatic index (HSI) of male mice. UPC2 -MS/MS analysis in both positive and negative mode, respectively, indicated significant differences between control and exposure groups in the liver of exposed mice. Principal component analysis (PCA) of the features revealed separation of control and exposure groups in both sexes. From the significantly differential 207 lipids, only 72 were identified and shown to belong to eight different lipid classes. PCA of fatty acids (FAs) profile showed a clear separation between control and PFAS exposure groups in both female and male mice, with differential abundant levels of 5 and 4 hydrolyzed FAs, respectively. Transcript and protein analysis of genes associated with lipid homeostasis (*ppar-α and β, lxr-α and β, rxr, fasn* and *srebp*) showed that PFAS exposure produced sex- and individual response related alterations. Glutathione reductase (Gr) activity showed exposure-related changes in both female and male mice, compared with controls. Overall, the present study has demonstrated changes in lipid metabolism after PFAS exposure, showing that PFAS accumulation in the liver resulted to hepatotoxic effects, potential interference with membrane lipid profile and homeostasis, and oxidative stress. Given the structural similarity with FAs, interaction between PFAS and nuclear receptors such as PPARs may have severe consequences for general health and physiology in exposed animals and humans.

1. Introduction

The regulation of metabolism and cellular energetics play integral roles for physiological functions and survival, and disruption of this balance often leads to chronic disease state (Metallo and Vander Heiden, 2013). Lipids are metabolized into fatty acids (FAs), which are energy-rich and ubiquitous biological molecules that are used for important cellular processes including as metabolic fuels, covalent regulators of signaling molecules, and essential components of plasma membranes (Forman et al., 1997). Further, FAs are signaling molecules that play important roles in regulating the expression of genes which mainly encode proteins for FA transport or metabolism (Duplus et al., 2000). The partitioning of cellular energetics into either oxidative or synthetic pathways represents critical steps in cell function and must be strictly coordinated (Wolfgang and Lane, 2006).

Contaminants induced alterations in cellular components, homeostasis and abundance of lipids will affect several biological processes in the body such as lipogenesis, lipid deposition and storage, lipid transport by lipoproteins, and FA uptake in tissues (Sheridan, 1988). The regulation of cellular FA pools is important for normal homeostasis (Forman et al., 1997) and nuclear factors such as the peroxisome proliferator-activated receptors (PPARs) are assumed to be master regulators of lipid homeostasis by controlling the balance between burning and storage of FAs (Shi et al., 2002). PPARs are ligand-dependent

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https://doi.org/10.1016/j.envint.2023.107838
Received 22 November 2022; Received in revised form 19 January 2023; Accepted 16 February 2023
Available online 18 February 2023

transcription factors of the classical nuclear hormone receptor superfamily (Shi et al., 2002). PPARs produce multiple effects by controlling energy homeostasis, adipose tissue differentiation and cellular maintenance and cell proliferation and tissue repair (Qi et al., 2000). Physiologically and through contaminant (such as PFAS) exposure, PPAR functions can be altered depending on several conditions that may be mediated through the ubiquitin–proteasome degradation system and extracellular signaling pathways and kinases, leading to receptor phosphorylation (Blanquart et al., 2003).

Per- and polyfluoroalkyl substances (PFAS) are persistent contaminants that bioaccumulate in the environment and biota (Baluyot et al., 2021). According to OECD/UNEP (2018), PFAS consists of over 4700 different compounds, where the predominant route of exposure is through food and drinking water for most organisms (Grønnestad et al., 2019). It has been shown that consumer products represent an integral source of PFAS exposure (Sunderland et al., 2019; De Silva et al., 2021; Grønnestad et al., 2021), despite the limited information about the effects of PFAS in consumer products on humans and wildlife. PFAS and their precursors have been manufactured for over 50 years and are ubiquitously used in several industrial and consumer products such as fluorinated polymers, surfactants, insecticides, ski wax and aqueous firefighting foams (Prevedouros et al., 2006). Although the production of some PFAS have been voluntarily phased out since 2002 (Martin et al., 2010), their levels in the biotic environment has continued to rise (Prevedouros et al., 2006; Houde et al., 2011). This is most likely due, in part, to continuing small scale productions in, for example, China and Brazil, but moreover due to large environment reservoirs of "PreFOS" such as PFOSA and *N*-alkylated-PFOSAs (Martin et al., 2010). There is limited to non-existent understanding on the time-dependent release, occurrence and accumulation patterns for most PFAS, in the environment and biota (McGuire et al., 2014). Further, the concept of mixture toxicity is not well considered for individual chemical-based on a paradigm that is often employed in various countries. Although there is known structural similarities among many PFAS, there is an almost complete lack of empirical knowledge on mixture toxicity for the ongoing simultaneous, chronic, low-level exposure to many known and unknown PFAS (Wang et al., 2017).

Most of the toxicological effects studies of PFAS on persistent perfluorocarboxylic acids (PFCAs) and Perfluoroalkyl sulfonates (PFSAs), have been derived from mammalian systems. For example, PFCA and PFSA and their precursors have been shown to exert a variety of toxicological effects, including – lipid homeostasis and peroxisome proliferation, oxidative stress, hepatomegaly, immunotoxicity, uncoupling of mitochondrial oxidative phosphorylation, developmental toxicity, reduction of thyroid hormone circulation, necrosis, down-regulation of hepatic transporters and tumors (Zheng et al., 2009; Austin et al., 2003; Ankley, 2005; Vanden Heuvel et al., 2006; Khan et al., 2020). Recent studies have highlighted PFAS effects on lipid metabolism. For example, Fragki et al. (Fragki et al., 2021) described PFOS and PFOA associated disruption of hepatocyte nuclear factor 4-alpha (HNF4α) pathway that is important for hepatic lipid homeostasis. Moreover, PFAS also interfere with mitochondrial fatty acid β-oxidation in the liver (Lu et al., 2019). PFAS exposure triggers steatosis through alteration of the balance between lipolysis and lipogenesis (Das et al., 2017), which also contributes in shifting metabolism from carbohydrate to fatty acid accumulation and oxidation (Bjork et al., 2021). We recently reported that PFAS significantly altered the dopamine (DA) system in both wild Bank voles from a skiing resort and A/J mice exposed in the laboratory (Grønnestad et al., 2019; Grønnestad et al., 2021). These effects on DA concentrations and associated homeostatic pathways contrasted in the two studies, and parallel molecular mechanisms of such effects (ibid).

Regardless of organism type, peroxisomes produce and degrade hydrogen peroxide (H_2O_2) and play significant roles in FAs metabolism (Lismont et al., 2019), through the PPAR signaling pathways. The increase in H_2O_2 production is due to exaggerated FA β-oxidation which may produce deleterious effects in organisms (Bonekamp et al., 2009). For example, superoxide dismutase (Sod), catalase (Cat) and glutathione peroxidase (Gpx) play important roles as antioxidants enzyme proteins, whose elevated expression and activity represent oxidative stress condition (Lushchak et al., 2001). Consequently, organismal adaptation during deprived oxygen condition requires the elevation of antioxidant and associated enzymes to reduce potential damage during oxygen reintroduction, such as lipid peroxidation (Storey, 1996). Therefore, the aim of our study is to evaluate the hepatic lipid homeostasis in A/J mice after dietary exposure to a PFAS mixture. We have chosen the PFAS composition and concentrations in the feed based on previous field data characterizing the occurrence, bioaccumulation, and biomagnification of PFAS in wild bank vole from a Nordic skiing area (Grønnestad et al., 2019). We hypothesize that exposure of A/J mice to relevant environmental concentration of dietary PFAS mixture will alter hepatic lipid profile and associated transcript and functional enzyme and protein products, and these effects will show sex-related differences in exposed individuals.

2. Materials and methods

2.1. Ethical considerations

The current experiment was conducted at the Section for Experimental Biomedicine, Norwegian University of Life Sciences (NMBU), in Oslo, Norway, by following national laws on the use of laboratory animals in research The Norwegian Food Safety Authority (https://www.mattilsynet.no/language/english/) has approved the facility and the experiment (application ID: FOTS 15446). The facility follows a health-monitoring program recommended by the Federation of European Laboratory Animal Science Association (FELASA, https://www.felasa.eu/) and the animals were kept under specific pathogen free (SPF) conditions.

2.2. Feed design and chemicals

The design of the PFAS mixture in the experimental feed was based on results from our previous study, where PFAS was analyzed in different matrices in a skiing area in Trondheim, Norway (Grønnestad et al., 2019). The used experimental concentrations were based on the highest concentration measured in earthworms for the most predominant PFAS deriving from ski wax since earthworms are part of the Bank voles' diet. Detailed exposure concentration and justifications is presented in Grønnestad et al. (Grønnestad et al., 2021).

2.3. Animals, husbandry, exposure and sampling

A/J mice bred in-house were used in the present study. At 3 weeks of age, whole litters were randomly assigned to either control or exposed group, resulting in 20 (10/10 males/females) and 18 mice (8/10 males/females) within the two groups, respectively. In order to create equal bioavailability of the compounds, the AIN-93G control or exposed pellet diets were provided *ad libitum* six days per week. The exposed and control gel diets were provided to the mice once per week (3 g/mouse) for the entire 10-week period, which corresponds to human years of 3–20 years old.

PFAS exposed and control mice were sacrificed at 13 weeks of age after a 10-week exposure period. Body weight was recorded prior to euthanasia by cardiac puncture and cervical dislocation under anesthesia (isoflurane gas obtained from Baxter, San Juan, Puerto Rico). Detailed exposure and sampling protocol is presented in Grønnestad et al. (Grønnestad et al., 2021) and S1 file.

2.4. Lipidomic analysis

Non-targeted lipid analysis of liver tissue was performed using an Ultrahigh-performance supercritical fluid chromatography (UPC2®)

coupled to a hybrid quadrupole orthogonal time-of-flight mass spectrometer SYNAPT G2-S HDMS (both Waters, Milford, MA, USA). The separation method was previously described (Lísa and Holčapek, 2015) and used with some modifications. Detailed protocol and chromatographic conditions and detection settings are presented in SI file.

2.5. RNA isolation and quantitative (real-time) polymerase chain reaction (qPCR)

Total liver RNA was isolated from frozen tissues using Direct-zol™ RNA extraction kit. Quality of RNA was confirmed by NanoDrop (see SI) and formaldehyde agarose gel electrophoresis and spectrophotometric analysis (see Figure S1 in SI). Transcript expression analysis related to lipid homeostatic pathways were performed using quantitative polymerase chain reaction (qPCR) with cDNA that was synthesized from 1 μg total RNA according to instructions provided with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). See SI Table 3 for gene-specific primer pair sequences and Khan et al. (Khan et al., 2019), for detailed qPCR protocol.

2.6. Western blotting analysis

Immunochemical analysis using the western blotting method was performed as described previously (Kortner et al., 2009), using specific antibodies for Ppar-γ, fatty acid synthase, ATP-citrate synthase, NF-κB and sterol regulatory-element binding proteins (Srebp) according to standard protocol. Detailed protocol is presented in the SI file.

2.7. Antioxidant enzyme assays

Hepatic antioxidant enzyme activities were measured in post-mitochondrial supernatant (PMS) from the control and exposed A/J mice according to standard protocols (Arukwe et al., 2015). Particularly, glutathione reductase (Gr) activity was determined spectrophotometrically by measuring NADPH oxidation at 340 nm (Wheeler et al., 1990), and specific activity was quantified using the extinction coefficient of 6.22 mM^{-1} cm^{-1}. Measured values were normalized against protein levels determined using the Bradford (1976) method.

2.8. Statistical analysis

Change in the abundance of lipid species (fold-change) was determined using Anova test provided by Progenesis QI inbuild statistical tools. Lipid profiles were analyzed by univariate and multivariate approaches using Metaboanalyst software (version 3.0). Score plots were constructed using abundance value of all peaks as selected by the software. Partial least square - discriminant analyses (PLS-DA) were performed to identify lipids that differed statistically between control and exposed mice. Heatmaps represents only those lipid species that differed statistically in control and PFAS exposed mice ($p < 0.05$).

Association between 16 lipid species whose VIPs > 1 and HSI were determined using Pearson correlation analysis considering the prerequisite of the test. Statistical analysis of transcripts and enzyme activity data were performed on IBM SPSS Statistics 25. The statistical differences between control and PFAS exposed mice were determined using one-way ANOVA (post-hoc Dunnett's test) at $\alpha = 0.05$. Normal distribution of data was performed using Shapiro-Wilk test. Variables that violated the normal distribution criteria were transformed using natural logarithm and square root transformation. In addition, applied Kolmogorov-Smirnov test to check normality was also performed. The homogeneity of variance was determined by Levene's test. Variables that violated Levine's homogeneity were investigated using Welch's heteroscedastic F-test and Brown-Forsythe test.

3. Results

3.1. PFAS accumulation and biometric measurements in mice

The relative liver weight to body weight (hepatosomatic index (HSI): liver weight/body mass*100) was significantly higher in males exposed to PFAS-mixture, compared to control males (two-way ANOVA, $p = 0.01$, $F = 6.35$) as reported in Grønnestad et al. (Grønnestad et al., 2021). Further, Grønnestad et al. (Grønnestad et al., 2021) also showed significantly higher concentrations of ∑PFAS (PFOA, PFNA, PFDA, PFUdA, PFDoDA, PFTrDA, PFTeDA and PFOS) in exposed mice liver compared to the control. The bioaccumulation of PFAS paralleled changes in body weight and hepatosomatic index (HSI) of male mice (Grønnestad et al., 2021).

3.2. Changes in liver lipidome and their functional characterization

Using UPC2 -MS/MS analysis in both positive and negative mode, respectively, showed 628 and 143 lipids in the liver of female mice (Table 1). Principal component analysis (PCA) of the features revealed separation of control and exposure groups in female (Fig. 1A). Out of 628 and 143 lipids, respective 208 and 73 fulfilled the set thresholds and showed significant differences between control and exposure groups. From the significantly differential 281 lipids, 95 were identified, belonging to eight (8) different lipid main classes (Table 1; SI Fig. 1). These classes belong to ceramides (Cer), diradylglycerols (DG), glycerophosphocholines (PC), glycerophosphoethanolamines (PE), glycerophosphoserine (PS), sphingomyelins (SM), sterols and triradylglycerols (TG) (SI Fig. 1).

For males, both positive and negative mode, respectively, showed 642 and 140 lipids (Table 1). PCA plot showed the separation of control and exposure groups (Fig. 1B). Out of 642 and 140 lipids, only respective 132 and 75 fulfil the set thresholds, showing significant differences between control and exposure groups. From the significantly differential 207 lipids, only 72 were identified, also belonging to eight (8) different lipids classes (Table 1; SI Fig. 2). These classes belong to ceramides (Cer), diradylglycerols (DG), glycerophosphocholines (PC), glycerophosphoethanolamines (PE), glycerophosphoserine (PS), sphingomyelins (SM), sterols and triradylglycerols (TG) (SI Fig. 2). Of the significant differentially identified lipids, both female and male share 54 lipid species, while 31 and 15 lipid classes were unique for female and male, respectively (SI Fig. 3).

The multivariate partial least-squares discriminant analysis (PLS-DA) demonstrate the lipid species that are responsible for the differences between control and exposure groups. Female and male mice have respective 13 and 9 species with variable importance in projection (VIP) value > 1 (Fig. 1C). These species belong to PC, TG and PE lipid class. Lipid species that are common between male and females include PC 38:4, PC 38:5, PC 38:6, TG 52:5, TG 56:5 and PE 38:6 (Fig. 1C). Unique lipid species in female are PC 34:2, PC 36:2, TG 54:3, TG 56:6, TG 58:8 and fragments of two TG species (represented with asterisk*). Unique lipid species in male include PC 36:4, TG 54:7 and TG 56:8 (Fig. 1C).

Table 1
Summary statistics of lipid species analyzed in mice liver using UPC2 -MS/MS at positive and negative modes.

Sex	Mode of operation	Total number of peaks picked	Significant differential levels of peaks between groups	Tentatively identified peaks	Total number of significantly different lipid species
Female	+ ve	628	208	81	95
	− ve	143	73	31	
Male	+ ve	642	132	61	72
	− ve	140	75	27	

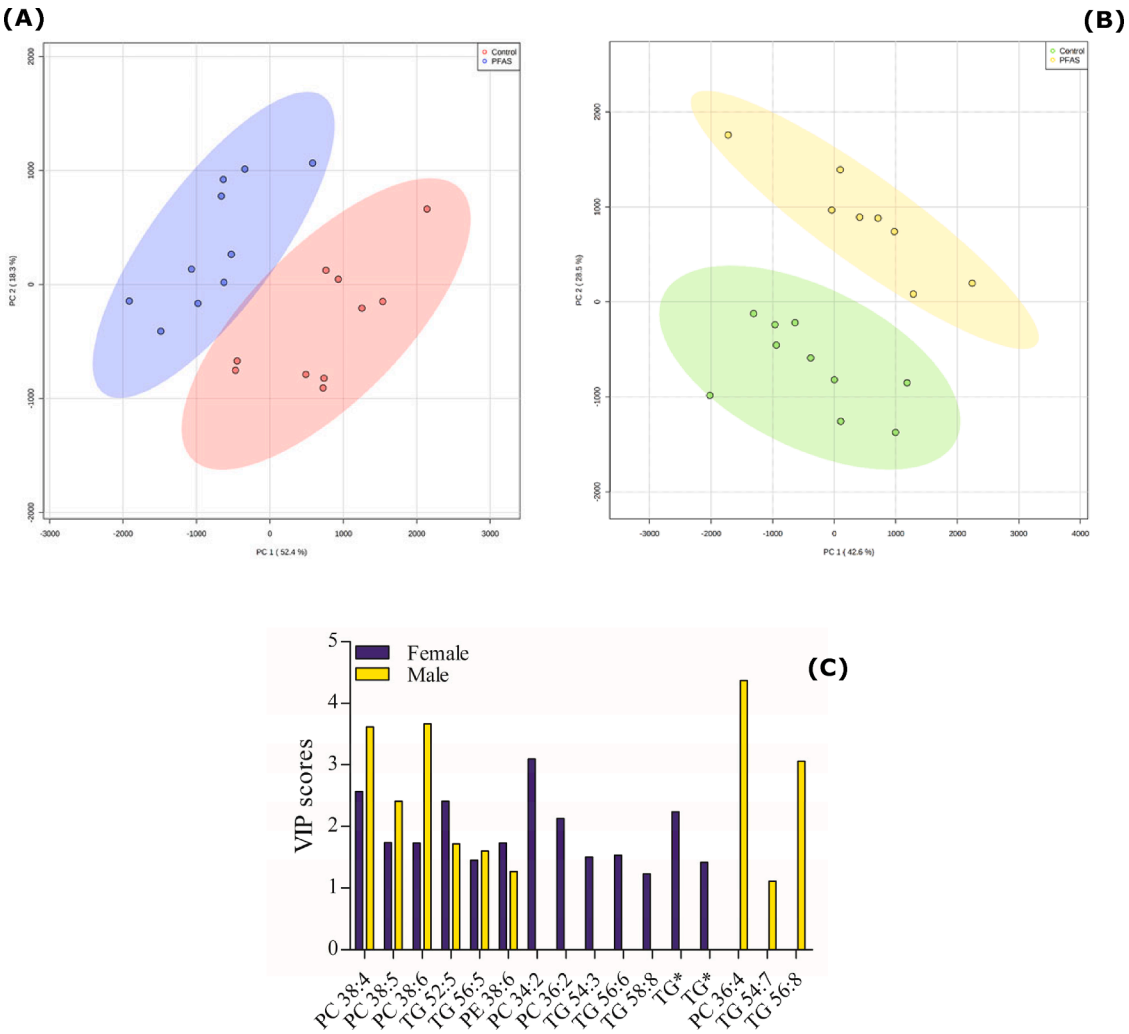

Fig. 1. Scores plot and Variable Importance in Projection (VIP) scores from PLS-DA multivariate analysis of liver lipidome in mice after PFAS exposure. Comparison in score plot between control and PFAS exposed female (A) and male (B) mice. Lipid species with VIP > 1 were considered relevant to explain the difference between control and PFAS-exposed mice, is represented in a bar plot (C). Blue and yellow bars represent female and male mice, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Functional characterization of differentially abundant lipid species using BioPAN indicate that pathway belonging to PC, PE and PS biosynthesis were significantly affected in mice liver after PFAS exposure (SI Table 1A and B).

3.3. Correlations between lipid species and HSI

We explored whether changes in lipid species after PFAS exposure is associated with the changes in HSI. Pearson's correlation analysis was performed between HSI and differentially abundant lipid species with VIPs > 1. In males, eight (8) out of nine (9) lipid species showed significant correlation with HSI. PC 36:4, PC 38:5 and TG 56:5 showed positive correlation, while on the other hand, PC 38:6, PE 38:6, TG 52:5, TG 54:7 and TG 56:8 was negatively correlated with HSI (Fig. 2A-B, SI Table 2). In female, only two (2) lipid species showed significant correlation, where TG 54:3 and TG 52:5 showed respective positive and negative correlation with HSI (Fig. 2C-D, SI Table 2).

3.4. Free fatty acids (FFAs) and FAs release from lipids

We optimized mass spectrometry procedure for the quantification of ten (10) FAs, namely - α-linolenic acid, docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), FA 16:1, FA 18:1, FA 20:1, FA 20:4, linoleic acid, palmitic and stearic acid and characterized their peaks with high confidence. PCA of these FAs showed a clear separation between control and PFAS exposure groups in both female and male mice (Fig. 3A). Female and male mice showed significant differential abundant levels of five (5) and four (4) hydrolyzed FAs, respectively. Among significantly differential FAs, four (4) are common between male and female mice, including - α-linolenic acid, DHA, DPA, FA 20:4. Only FA 18:1 is present at significantly high level in female mice (Fig. 3B). In addition to hydrolyzed FAs, some free FAs were also identified in the negative mode, while analyzing the lipid species. Like hydrolyzed FAs, DHA was identified as a free FA in the negative mode in both male and female mice. Besides DHA, arachidonic acid as a free FA was also present at significant differential level in male mice.

3.5. Transcript expression

We analyzed transcript (*ppar-α and β, liver × receptor (lxr-α and β)), retinoid × receptor (rxr), fatty acid synthase* (*fasn*) and *srebp*) levels of genes associated with lipid homeostasis in female and male A/J mice after exposure to PFAS mixture. In male mice, PFAS exposure did not produce changes in *ppar-α* transcript, while a significant reduction was observed in PFAS exposed females, compared with control (Fig. 4A and B, respectively). For *ppar-β*, PFAS exposure produced significant

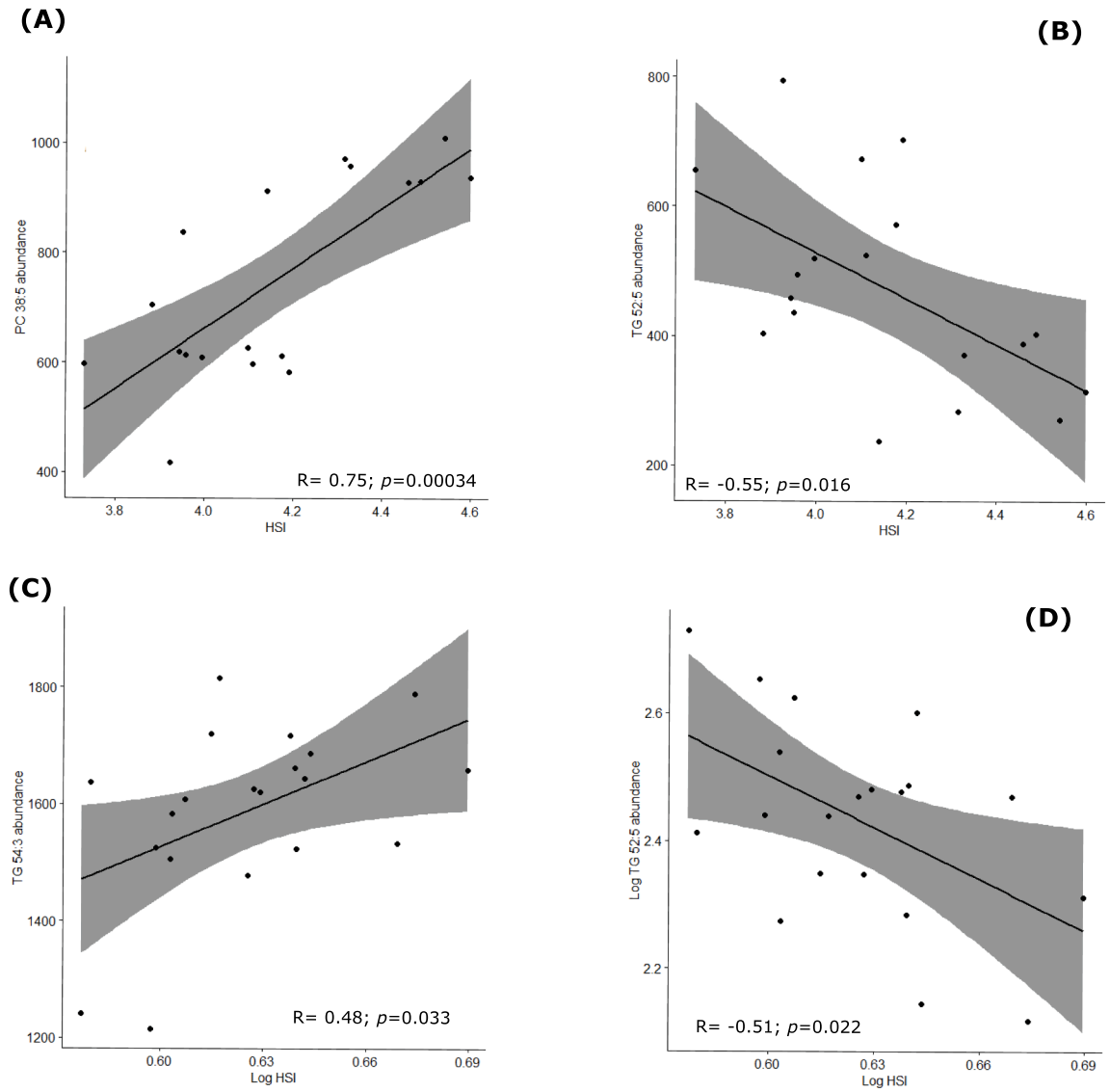

Fig. 2. Pearson's correlation between hepatosomatic index (HSI) and abundance value of differentially abundant lipid species with VIPs > 1. In female mice, out of eight significant correlated lipid species, only PC 38:5 (A) and TG 52:5 (B) is represented in the figure. In males, TG 54:3 (C) and log-transformed TG 52:5 (D) significantly correlated with HSI.

elevation of transcript levels in both male and female mice (Fig. 4C and D, respectively). Male mice exposed to PFAS produced significant increase in *lxr-α* transcript, while no effect was observed in females, compared with control (Fig. 4E and F, respectively). For *fasn, lxr-β, srebp and rxr,* no significant PFAS related changes in transcript levels were observed in both male and female A/J mice, compared to controls (SI Fig. 4A-H, respectively).

3.6. Protein expression

Immunochemical analysis of protein expression pattern in A/J mice after PFAS exposure showed apparent sex-related effects on protein abundance level for Ppar, Fasn, Srebp, NF-κB and ATP-citrate synthase (SI Fig. 5–8, respectively). Ppar protein expression showed higher abundance in control mice compared with PFAS exposed female group (SI Fig. 5A), while no differences were observed in male mice (SI Fig. 5B). Minor, but non-significant changes were observed in Fasn protein abundance between exposed groups and sex groups (SI Fig. 5C and D). For Srebp and NF-κB, significant elevation in protein abundance was for observed for pre-Srebp1 (p-Srebp1), matured-Srebp1 (m-Srebp1) and NF-κB, higher in PFAS exposed female and male (SI Fig. 6 and 7) mice, compared with controls. ATP-citrate synthase protein expression showed higher abundance in PFAS exposed female mice compared with control group (SI Fig. 8), while no differences were observed in male mice (SI Fig. 8).

3.7. Antioxidant enzymes

Among the analyzed antioxidant enzymes (Gr, Gpx, Sod and Cat), only the Gr showed exposure-related changes in enzyme expression levels with significant elevation after PFAS exposure in both female and male mice, compared with controls (Fig. 5A and B, respectively).

4. Discussion

Available evidence suggests that PFAS, and related organic contaminants may alter cellular lipid synthesis, metabolism and associated signaling pathways in several higher and lower vertebrate species, potentially leading to the disruption of lipid homeostasis and oxidative stress. Herein, we have studied the hepatocellular lipid profile and

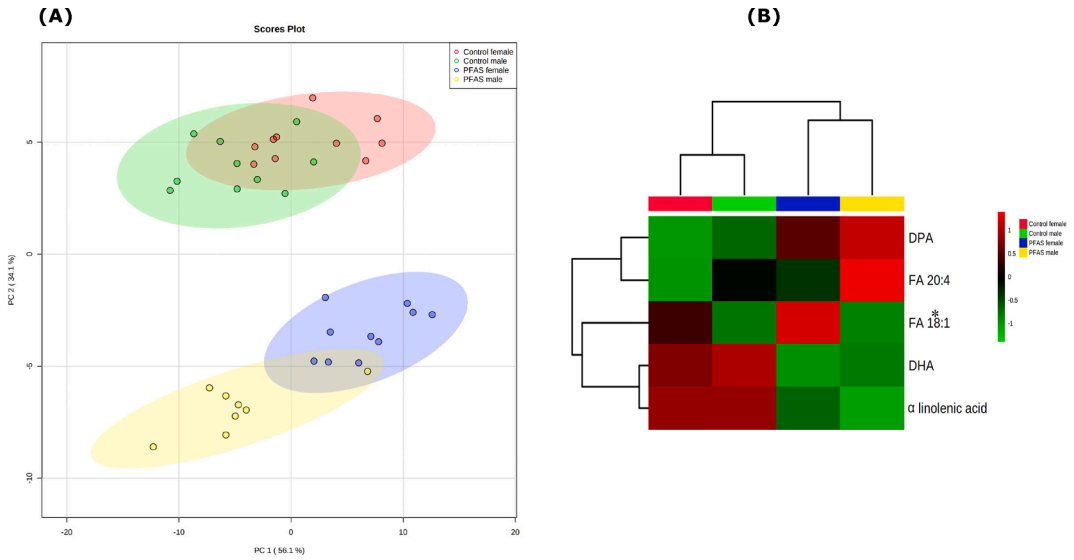

Fig. 3. Scores plot and heat map of hydrolyzed fatty acids (FAs) in mice liver after PFAS exposure. Comparison in score plot between control and PFAS exposed male and female mice (A). The heatmap (B) represents hydrolyzed FA levels, with green and red representing low and high abundance, respectively. Asterisks (*) indicate hydrolyzed FA that is abundant at significantly higher level in female mice exposed after PFAS exposure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

associated functional products in A/J mice after exposure to environmental relevant concentration of PFAS mixture. Thus, the present work explored change in the metabolism of lipids after PFAS exposure, demonstrating that the liver accumulated PFAS resulting to hepatotoxic effects, interference with membrane lipid profile and homeostasis, and oxidative stress. Given the structural similarity with FAs, interaction between PFAS and nuclear receptors such as PPARs is inevitable and will disrupt hepatic lipid metabolism.

4.1. *Lipidomics and functional characterization*

UPC2 -MS/MS analysis demonstrated several hepatic lipid peaks in both female and male mice, and a PCA evaluation revealed clear and significant separation of these lipid classes into control and exposure groups in both female and male mice. Using the same A/J mice as in the current study, Grønnestad, Johanson (Grønnestad et al., 2021) observed increase in liver HSI and body mass and these changes paralleled PFAS liver bioaccumulation (*ibid*). Previously, Das, Wood (Bassler et al., 2019) showed that wild-type (WT) mice treated with perfluoroalkyl acids (PFAA) induced significant increase of absolute liver weights, as well as lipid accumulation in the liver. Elsewhere, it was reported that PFAS treated WT mice and *ppar-α*-null mice showed exposure-related expression of transcripts for lipid catabolism, together with those involved in FA and triglyceride (TG) synthesis, suggesting that PFAA congeners may alter hepatic TG abundance and drive the development of steatosis in mice (Das et al., 2017).

Furthermore, respective increase and decrease in cell number and size after exposure to PFAS, and associated decrease in the volume fraction of average hepatic cellular lipid levels (Bassler et al., 2019) can be attributed to cellular adaptation to exposure condition. The authors also reported that these physiological alterations paralleled changes in transcripts expression patterns for a group of genes involved in lipid homeostasis in an apparent compound- and concentration-specific manner (Fragki et al., 2021). These relationships between PFAS exposure and hepatotoxicity mentioned above are in accordance with our findings showing that TG and PC levels were significantly related to HSI in exposed A/J mice. In the minimum, it is possible that the increase in HSI might be an adaptive cellular response by increasing cell number (hyperplasia) and size (hypertrophy) to tackle persistent PFAS burden (Agency, 2016). Nevertheless, given that the mice did not generally gain weight during the exposure period, and only the males had elevated HSI, it is possible that PFAS altered gluconeogenesis and insulin resistance leading to flux of lipids from adipose tissue to the liver. The argument for this is that lipid retention in the hepatocytes reduces cellular functions leading to liver adaptation with hyperplasia to compensate for PFAS-mediated disruption.

However, given that the increase in HSI paralleled lipid levels in the liver, we propose that this observation represent a novel aspect of PFAS hepatotoxicity. This observation and novelty are supported by previous reports showing that in small mammals, the liver is an integral and primary target organ for PFAS exposure both at acute and chronic scenarios (Cui et al., 2009; Dong et al., 2012; Yu et al., 2016). Furthermore, it has been reported that PFOS and PFOA increased liver weight relative to bodyweight and hepatic peroxisomal β-oxidation in small mammals at respective 0.15- and 0.64 mg/kg/day (EFSA, 2018). We are mindful that the doses used by the European Food Safety Authority (EFSA, 2018) are several orders of magnitude higher than the doses used in our study. Nevertheless, both studies provide significant understanding that PFAS, when given individually or in combination may potentially produce hepatotoxic effects in exposed animals (Grønnestad et al., 2021). The liver is the primary organ for the metabolism of endogenous and exogenous substances. For example, carbohydrates, fatty acids and protein are all processed mainly in the liver, making it the major organ for metabolism of these substances. Most PFAS are amphiphilic, resulting to their partitioning in protein-rich tissues, and the liver has been identified as the main organ for PFAS accumulation in mammals (Aas et al., 2014), rendering the liver as a natural target for PFAS toxicity (EFSA, 2018). Furthermore, PFAS are known to increase liver weight or liver enlargement (hepatomegaly) and overexpression of fatty acid β-oxidation-related genes both *in vitro* and in experimental animal models. Given that the sex differences in the portioning and composition of lipids and proteins in animals, all these physiological processes may explain the sex-related differences observed in HSI of exposed animals.

Several classes of lipids, including 20 and 22 carbon polyunsaturated fatty acids (PUFAs) are integral parts of membrane phospholipids, and with important roles in several steps during cell signaling, control the expression of many transcripts involved in lipid synthesis and metabolism, thermogenesis, and cell differentiation (Papackova and Cahova, 2015). Despite these, a significant number of previous investigations on the alteration of FA metabolism after exposure to PFAS have mostly

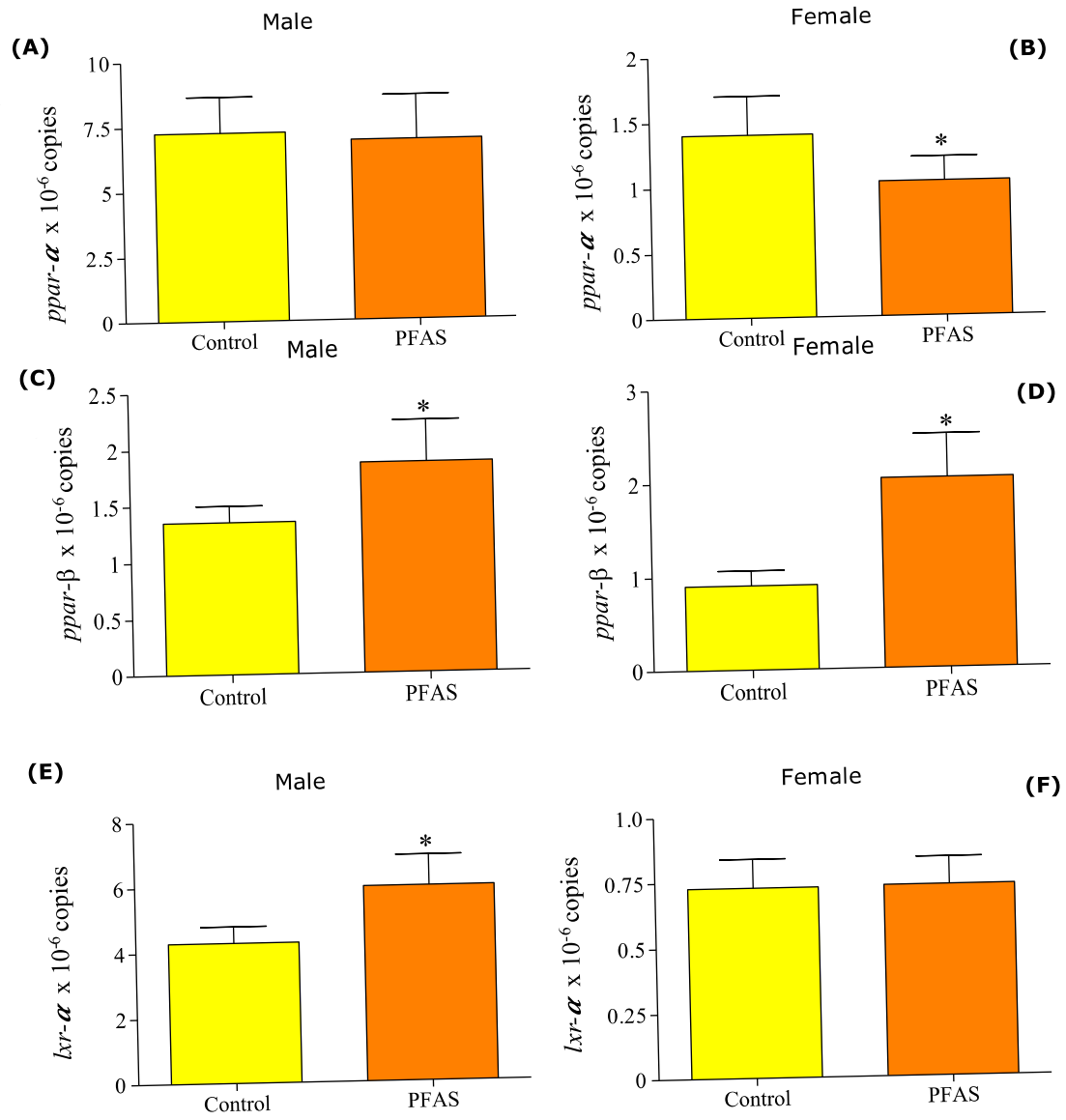

Fig. 4. Change in transcript levels of *ppar-α* (A, B), *ppar-β* (C, D), and *lxr-α* (E, F) genes in mice liver after PFAS exposure. The expression levels are presented as mean transcript copies $\times 10^{-6}$ of n = 20 and 18 for females and males, respectively. Data are presented as mean values ± SE. Asterisk (*) indicates statistical significance ($p < 0.05$) between the control and PFAS exposure group.

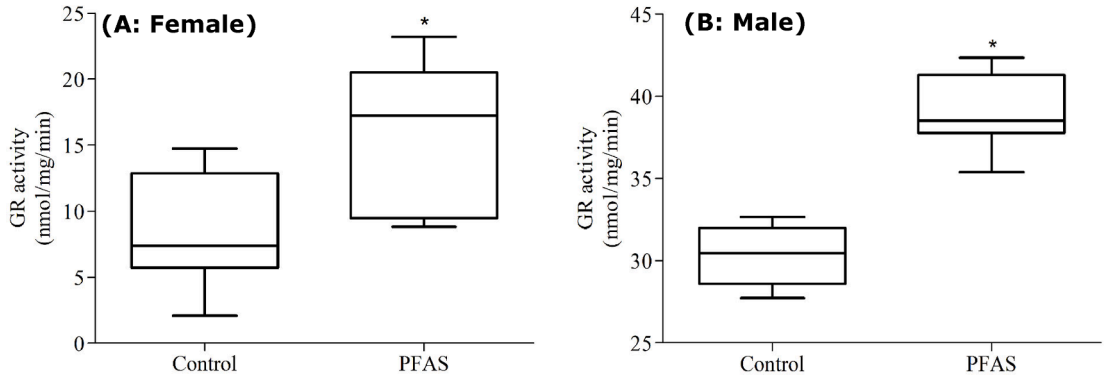

Fig. 5. Glutathione reductase (Gr) activity in the liver of mice exposed to PFAS. The activity is represented as nmol/min/mg protein in female (A) and male (B) mice with median values and 25–75% quartiles and whiskers that are calculated using Tukey's test. Asterisk (*) shows statistical significance ($p < 0.05$).

focused on β-oxidation (Chen et al., 2020). Herein, we showed that PFAS altered FA profiles, including DPA, DHA and α-linolenic acid (α-LA) (Wågbø et al., 2012). Eicosanoic acids or eicosanoids are key regulators of a wide variety of physiological responses and pathological processes, and control important cellular processes (Calder, 2020). Arachidonic acid [ARA: 20:4n-6] is produced from linoleic acid (LA) as precursor through elongation and saturation (Tallima and El Ridi, 2018). Eicosanoids such as prostaglandins, thromboxanes and leukotrienes are derived from ARA and EPA (Bell et al., 2003), and act primarily as potent local cellular regulators (Cave, 1991). In mammals, overproduction of eicosanoids derived from ARA may potentially produce several pathophysiological conditions, such as atherothrombotic and chronic inflammation diseases (Dennis and Norris, 2015). When these pathophysiological conditions are viewed in the context of our data, it strongly suggests that PFAS mediated changes in lipid class levels are possibly compensated for lipid peroxidation by altering PPAR signaling (see discussion below) through elevated elongation and desaturation processes to increase membrane fluidity as compensatory mechanism or possible precursor to other disease, physiological (including endocrine effects) and pathological states (Deierlein et al., 2017; Behr et al., 2020).

4.2. Alteration of lipid signaling pathways

In mammals, as in other vertebrate species, lipid homeostasis is regulated through the ppar signaling pathways. Herein, we measured the expression of *ppar* isoforms and their dimeric partners, both at transcript and functional product (protein) levels, showing that PFAS exposure produced gender-related changes in *ppar-α, ppar-β and lxr-α* expression patterns. However, no related significant changes were observed for *srebp, NF-κB, ATP-citrate synthase, fasn, lxr-β* and *rxr* at transcript level, while these variables showed gender and PFAS exposure related changes at protein level. For example, the expression of *ppar-β* has been demonstrated to display a wide tissue distribution pattern, although its isoform-specific physiological functions are not fully characterized. However, in mammals, it has been suggested that *ppar-β* participates in the overall physiological control of lipid homeostasis (Peters et al., 2012), and in the absence of ligands, may function as *ppar-α* and *ppar-γ* repressor (Ricote and Glass, 2007). Previous studies have demonstrated that PFAS have positively altered the expression of genes (and their functional products) involved in peroxisomal β-oxidation (Jacobsen et al., 2018). Our findings are in accordance with previous report (Liu et al., 2008) showing that PFAS produced *trans*-activation of *ppar-α* in transfected COS-1 cells. Elsewhere, it has been shown that the differential expression patterns in transcript and/or functional products of ppar isoforms may eventually produce a disruption of normal energetics and its homeostatic control in exposed organisms. Using an *in vitro* assay system with mammalian ligand binding domains of either *ppar-α* or *ppar-γ*, Evans, Conley (Evans et al., 2022) evaluated a suite of PFAS and fluorotelomer alcohols (FTOHs) activated these *ppar* isoforms, showing that all, except the FTOHs and PFOSA, activated these *ppar* isoforms.

Elsewhere, it has been reported that organotins such as tributyltin (TBT) are classical *ppar* and *rxr* ligands (forming the *rxr–ppar* heterodimer) (Grün and Blumberg, 2006; Nakanishi, 2008). In mammalian system, it was shown that TBT activated the heterodimeric formation between *rxr* and *ppar-α, β* and *γ,* mainly through *rxr* interaction (Le Maire et al., 2009; Kanayama et al., 2005). Recently, Papadopoulou, Nicolescu (Papadopoulou et al., 2022) evaluated the levels and composition of lipoproteins major subclasses in 127 plasma samples from adult participants of the EuroMix human biomonitoring study using nuclear magnetic resonance (NMR) technology. The authors reported that serum levels of 17 PFAS demonstrated an occurrence frequency between 30 and 100%, and adjusted model revealed positive associations between several PFAS and cholesterol concentrations in high-density lipoprotein (HDL) and low-density lipoprotein (LDL) particles. In addition, Papadopoulou, Nicolescu (Papadopoulou et al., 2022) demonstrated that HDL cholesterol per interquartile range (IQR) increased in parallel with several PFAS. Particularly, the associations with PFNA, PFUnDA, PFDoDA and PFOS were significant in women after adjustment for multiple comparisons and a change in magnitude between longer chained PFAS and LDL cholesterol. In another study, a positive relationship was observed between HDL cholesterol and plasma PFAS concentrations, and between PFOS and total cholesterol in samples from pregnant women in Norway (Starling et al., 2014).

Ammonium 2,3,3,3-tetrafluoro-2-(heptafluoropropoxy) propanoate (GenX) was shown to induce comparable expression pattern of genes involved in lipid, monocarboxylic acid, and ketone metabolisms at 0.1 and 10 μM concentration, and differentially so at 100 Mm (Robarts et al., 2022). Among the 576 and 375 transcripts with respective positive and negative relationship with GenX concentration, the upstream regulator analysis indicated that *hif1α* was reduced at lower GenX concentrations but elevated at the higher concentration, while *vegf, ppar-α, stat3*, and *smad4* signaling were increased at the 100 μM concentration (Robarts et al., 2022). Collectively, these findings and our current data show that PFAS are potent modulators of cellular lipid profile, associated homeostasis and signaling pathways. On the other hand, ppar-α displays high hepatic expression profile (Shi et al., 2002), which controls the expression of genes that play integral roles in several physiological processes such as inflammation, lipid metabolism and cellular differentiation and growth (Rosen et al., 2009). Furthermore, the regulation of FA β-oxidation (Liu et al., 2008) and subsequent production of reactive species such as hydrogen peroxide (H_2O_2) and leading to oxidative stress and damage (Hu et al., 2005) is controlled by *ppar-α*. Relevant to this, we observed that PFAS exposure significant induced the activity of Gr in both male and female mice.

4.3. Alteration of oxidative stress responses

Contaminants and other environmental stressors may alter the balance between ROS production and antioxidants scavenging with possible direct or indirect effects on physiological functioning of cells and cellular macromolecules such as DNA, protein, and lipids (Kamat et al., 2000). Under normal physiological conditions, H_2O_2 is a substrate for Gpx enzymes, where reduced glutathione (GSH) is the electron donor in a reaction that catalyzes the reduction of H_2O_2 to H_2O. Thus, Gpx enzymes, together with some GST isoenzymes, reduce lipid hydroperoxides to alcohol, and subsequently oxidizing GSH to GSSG (Regoli and Giuliani, 2014). While Gr is not a classical antioxidant enzyme, it reconverts oxidized GSH, thereby playing integral role in maintaining normal physiological GSH/GSSG ratio and intracellular redox status in organisms (Regoli and Giuliani, 2014). In this study, we analyzed Gr, Gpx, Sod and Cat, and only the Gr showed exposure-related changes at functional enzyme levels with PFAS-induced significant increase in female and male mice. For example, Cat controls the acquisition of tolerance to oxidative stress as cellular adaptive response against severe oxidative stress (Mates, 2000) that include reduction of hydrogen peroxide (H_2O_2) to H_2O in the peroxisomes.

PFAS such as PFOA was reported to elevate functional responses for antioxidant enzymes such as Cat, Sod and Gr in fish hepatocytes (Liu et al., 2008). Elsewhere, a consistent relationship between PFAS and biomarkers of oxidative stress measured as malondialdehyde, hydrogen peroxide) has been reported in humans and mice (Omoike et al., 2021). Furthermore, a correlation between depletion of intracellular GSH concentration and cytotoxicity was observed in human liver cells (HepG2) exposed to PFAS (Ojo et al., 2020; Ojo et al., 2021), suggesting that PFAS, given individually or in combination could induce concentration-dependent cytotoxicity and depletion of GSH levels, without significant elevation of ROS production (Ojo et al., 2021). The present data are in accordance with previous data showing that exposure of mammals to PFAS produced alterations in toxicological variables related to oxidative stress responses and lipid peroxidation (TBARS) and that these responses are generally dependent on organ, association with

tissue bioaccumulation patterns and dependent on exposure (O'Brien et al., 2005; Arukwe and Mortensen, 2011; O'Brien et al., 2001). Thus, *in vitro* and *in vivo* experiments using fish and mammals suggest that increases in ROS production may elevate oxidation of fatty acids and lipid peroxidation, with potential organ-selective metabolic and physiological consequences.

5. Conclusions

The present study has demonstrated changes in lipid homeostasis after PFAS exposure, showing that the liver accumulated PFAS resulting to hepatotoxic effects (increase in HSI), interference with membrane lipid profile and overt homeostasis, and oxidative stress. PFAS exposure may ultimately increase steatosis due to the disruption of the balance between fatty acid accumulation/synthesis and oxidation to favor accumulation. With respect to overt consequences for toxicological, physiological, and general health context, our findings are relevant and in accordance with previous studies reporting several effects of organic and inorganic contaminants on lipid and FA homeostasis, particularly in ARA, in several species, including humans (Borlakoglu et al., 1991; Dorman and Freeman, 2002; Kawashima et al., 1989; Knowles and Donaldson, 1996; Ulberth, 2000). PFAS altered ARA levels, which is released from phospholipids and transformed to several eicosanoids such as prostaglandins that are involved in steroidogenesis through the cyclooxygenase and lipooxygenase pathways. These effects may account for the gender-related differences observed in the present study and generally produce deleterious physiological and endocrinological consequences, with direct relevancy to human health. Relevant to this, prostaglandin synthesis is collectively controlled by different enzymes including phospholipase, cyclooxygenase, lipoxygenase and ARA content of phospholipids (Morita et al., 1983). In addition, contaminants-mediated cell damage and related oxidative stress may depend largely on the type of FAs present in cell membrane fluidity and enable contaminants transport across plasma (Prasad et al., 2010).

CRediT authorship contribution statement

Essa A. Khan: Formal analysis, Writing – original draft, Writing – review & editing. **Randi Grønnestad:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing. **Åse Krøkje:** Conceptualization, Funding acquisition, Writing – review & editing. **Zdenka Bartosov:** Formal analysis, Writing – review & editing. **Silje Modahl Johanson:** Investigation, Writing – review & editing. **Mette H. B. Müller:** Investigation, Writing – review & editing. **Augustine Arukwe:** Conceptualization, Funding acquisition, Investigation, Supervision, Project administration, Resources, Writing – original draft, Writing – review & editing.

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.

Acknowledgments

We thank the Mass Spectrometry Laboratory at the Faculty of Natural Science, NTNU, Norway where the UPC2 -MS/MS lipidomic analysis was performed. We would like to acknowledge Diana Denzil Rozario for assistance in lipid extraction. We also thank Susana Villa Gonzalez for help with data processing and lipid identification. Financed by NTNU (DVA).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2023.107838.

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