Research Article
NAFLD and Alcohol-Related Liver Diseases

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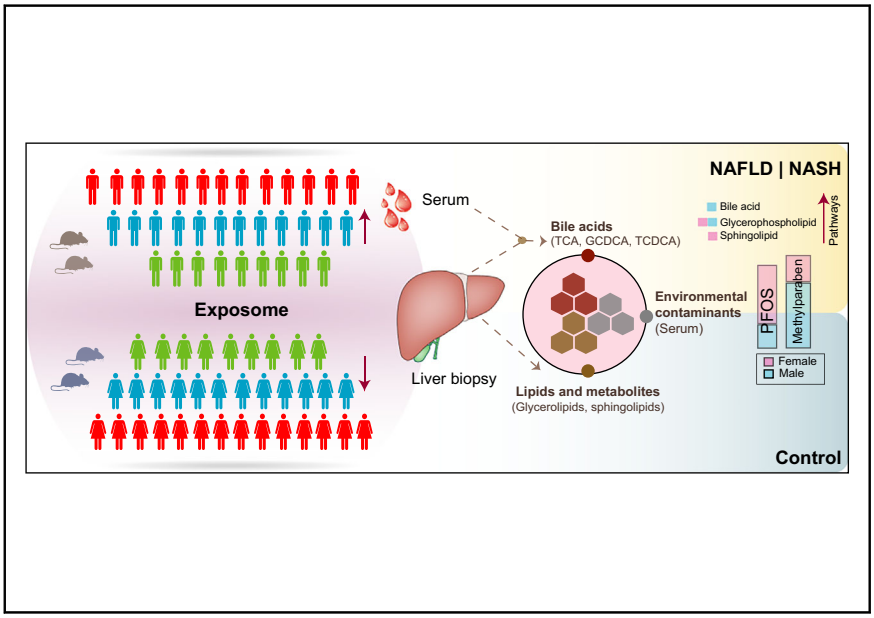
Exposure to environmental contaminants is associated with altered hepatic lipid metabolism in non-alcoholic fatty liver disease

Graphical abstract



Highlights

- Environmental contaminants may contribute to the initiation and development of NAFLD.
- Exposure to PFAS is associated with the alteration of bile acid profiles and NAFLD-related pathways in the human liver.
- Other lipid-related changes may be secondary to the interplay between PFAS and bile acid metabolism.
- Females may be more sensitive to the harmful impacts of PFAS than males.

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Lay summary

There is increasing evidence that specific environmental contaminants, such as perfluorinated alkyl substances (PFAS), contribute to the progression of non-alcoholic fatty liver disease (NAFLD). However, it is poorly understood how these chemicals impact human liver metabolism. Here we show that human exposure to PFAS impacts metabolic processes associated with NAFLD, and that the effect is different in females and males.

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Exposure to environmental contaminants is associated with altered hepatic lipid metabolism in non-alcoholic fatty liver disease

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Background & aims: Recent experimental models and epidemiological studies suggest that specific environmental contaminants (ECs) contribute to the initiation and pathology of non-alcoholic fatty liver disease (NAFLD). However, the underlying mechanisms linking EC exposure with NAFLD remain poorly understood and there is no data on their impact on the human liver metabolome. Herein, we hypothesized that exposure to ECs, particularly perfluorinated alkyl substances (PFAS), impacts liver metabolism, specifically bile acid metabolism.

Methods: In a well-characterized human NAFLD cohort of 105 individuals, we investigated the effects of EC exposure on liver metabolism. We characterized the liver (*via* biopsy) and circulating metabolomes using 4 mass spectrometry-based analytical platforms, and measured PFAS and other ECs in serum. We subsequently compared these results with an exposure study in a PPARa-humanized mouse model.

Results: PFAS exposure appears associated with perturbation of key hepatic metabolic pathways previously found altered in NAFLD, particularly those related to bile acid and lipid metabolism. We identified stronger associations between the liver metabolome, chemical exposure and NAFLD-associated clinical variables (liver fat content, HOMA-IR), in females than males. Specifically, we observed PFAS-associated upregulation of bile acids, triacylglycerols and ceramides, and association between chemical exposure and dysregulated glucose metabolism in females. The murine exposure study further corroborated our findings, *vis-à-vis* a sex-specific association between PFAS exposure and NAFLD-associated lipid changes.

Conclusions: Females may be more sensitive to the harmful impacts of PFAS. Lipid-related changes subsequent to PFAS exposure may be secondary to the interplay between PFAS and bile acid metabolism.

Lay summary: There is increasing evidence that specific environmental contaminants, such as perfluorinated alkyl substances (PFAS), contribute to the progression of non-alcoholic fatty liver disease (NAFLD). However, it is poorly understood how these chemicals impact human liver metabolism. Here we show that human exposure to PFAS impacts metabolic processes associated with NAFLD, and that the effect is different in females and males.

Introduction

The liver plays a vital role in the maintenance of metabolic homeostasis, whilst also being a key organ involved in the metabolism, distribution, and excretion of exogenous chemicals. As hepatocytes are exposed to a significant influx of various exogenous chemicals, chemical-induced hepatotoxicity is a worldwide health concern. Indeed, hepatotoxicity is a common endpoint in the risk assessment of many environmental contaminants (ECs), including endocrine-disrupting chemicals (EDCs).[1]

Recent data suggest that hepatic steatosis associates with exposure to toxic EDCs.[2,3] Exposure to persistent ECs may also initiate and promote the pathogenesis of non-alcoholic fatty liver disease (NAFLD).[4,5] EDCs may act as a 'second hit' in the progression of NAFLD, driving the disease from an earlier, less severe stage, such as steatosis, to the more severe stages such as non-alcoholic steatohepatitis (NASH). Alternatively, exposures to EDCs may also represent the 'first hit', which compromises the liver's protective responses against over-nutrition, predisposing it to steatohepatitis following a subsequent 'hit' from a hypercaloric diet.[3]

A specific class of EDCs linked with NAFLD are perfluorinated alkyl substances (PFAS). These are synthetic chemicals used for

Keywords: chemical exposure; exposome; perfluorinated alkyl substance; non-alcoholic steatohepatitis; fibrosis; bile acid; lipidome; metabolome; metabolic pathway.
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various industrial applications and in consumer products. As PFAS are highly persistent in the environment, the general population is widely exposed to these substances, mainly through diet and contaminated water. In human epidemiological and animal toxicology studies, PFAS exposure has been identified as associated with a variety of adverse health outcomes.[6] Based on studies in experimental models, PFAS gradually accumulate in the liver where they are highly hepatotoxic, interfering with glucose and lipid metabolism, elevating liver enzymes, and exacerbating the effect of a high intake of dietary fat.[4] Whilst there are some human studies linking PFAS exposure to changes in the circulating metabolome,[6] there are currently no data on the impact of PFAS exposure on the human liver metabolome.

Due to the structural similarity between PFAS and fatty acids, PFAS may disrupt hepatic lipid metabolism by interacting with receptors such as peroxisome proliferator-activated receptors (PPARs) and other nuclear receptors, including the constitutive androstane receptor and the pregnane X receptor.[7] PFAS may also promote steatosis by upregulating lipogenesis and lipid influx to the liver, whilst downregulating liver lipid efflux.[3] Particularly, PFAS interfere with bile acid (BA) synthesis, and several steps of their enterohepatic circulation.[8,9] BAs are a specific class of lipids synthesized in the liver from cholesterol, with regulatory roles in metabolic and cellular homeostasis[10]; BAs have been reported to be increased in the liver tissue,[11,12] plasma,[11,13,14] and feces[13] of patients with NAFLD.

The conclusions from various human epidemiological studies concerning the possible link between PFAS exposure and cardiometabolic disorders (including NAFLD) remain inconsistent.[15] A plausible explanation for these inconsistent findings is that the epidemiological studies insufficiently account for individual biological factors, including internal exposures, such as the metabolome, which are likely to have a major impact on human health.[6] Sex differences also have generally not been sufficiently accounted for in such studies. Sex has a major impact on lipid (including BA) metabolism, with sex-based differences reported in exposure studies in animal models.[16,17]

Herein, we hypothesized that exposure to ECs, including PFAS, impact liver metabolism, specifically BA and lipid metabolism. In a well-characterized human NAFLD cohort of 105 individuals,[18] we investigated the impact of EC exposure on liver metabolism. We characterized both liver (*via* biopsy) and circulating metabolomes using 4 analytical platforms, and measured PFAS and other ECs in serum. In order to elucidate any causal relationships between PFAS exposure and specific metabolic pathways, we subsequently compared these results with a PPARa-humanized mouse model exposed to perfluorooctanoic acid (PFOA), one of the most widely detected PFAS in humans.

Patients and methods

Study participants

A total of 105 patients (70 female, 35 male) were recruited from those undergoing laparoscopic bariatric surgery. Patients were eligible if they met the following criteria: i) age 18 to 75 years; ii) no known acute or chronic disease except for obesity, type 2 diabetes or hypertension as assessed by medical history, physical examination and standard laboratory tests (complete blood count, serum creatinine, electrolyte concentrations); iii) alcohol consumption <20 g per day for women and <30 g per day for men; iv) no clinical or biochemical evidence of other liver disease, or clinical signs or symptoms of inborn errors of metabolism; v) no history of use of toxins or drugs associated with liver steatosis. Elevated liver enzymes (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) were not exclusion criteria. Clinical measurements were performed as reported previously[18] (see also the supplementary methods). Liver histology was analyzed by an experienced liver pathologist (J.A.) in a blinded manner, as proposed by Brunt *et al.*[19] Macrosteatosis was assessed visually as the percentage of hepatocytes occupied by macrovesicular lipid droplets (*i.e.*, large intracytoplasmic lipid droplets displacing the nucleus to the cell's periphery). Necroinflammatory activity was graded from 0 to 3 and fibrosis stage from 0 to 4.[19]

Murine exposure study

Data from this murine study were previously reported.[20] A brief summary is provided in the supplementary methods.

Analyses of metabolites and environmental contaminants

Multiple analytical protocols were used for analysis of metabolites and ECs, as described in detail in the supplementary methods and supplementary CTAT table; BAs and PFAS in serum by ultra-high-performance liquid chromatography quadrupole time-of-flight mass spectrometry method (UHPLC-QTOFMS), hepatic polar metabolites (two-dimensional gas chromatography coupled to TOFMS; GC×GC–TOFMS), hepatic molecular lipids (UHPLC-QTOFMS), hepatic acyl-carnitines (UHPLC coupled to triple-quadrupole MS; UHPLC-QQQMS), hepatic BAs (UHPLC-QQQMS).

Statistical analyses

All the metabolomics, lipidomics and the environmental toxins (ECs) data were $\log_2$ transformed. Homogeneity of the samples were assessed by principal component analysis.[21] The R statistical programming language (v.3.6.0)[22] was used for data analysis. Statistical methods are described in detail in the supplementary methods.

Results

Metabolic and chemical exposure profiles in the liver and serum showed large biological variation

Patients in this study (n = 105) were obese (mean BMI = 46±6 kg/m^2) with a liver fat content between 0% to 80% (Tables S1 and S2). We analyzed metabolic profiles in the liver, while the ECs were measured only in serum due to the limited sample amounts of human liver biopsies. However, PFAS levels in circulation closely reflect concentrations in the liver.[23]

Polar metabolites such as free fatty acids and amino acids involved in gluconeogenesis and central carbon metabolism, were measured quantitatively, while molecular lipids were measured semi-quantitatively using calibration by lipid class-specific internal standards. Multiple classes of lipids, including cholesterol esters, lysophosphatidylcholines (LPCs), lysophosphatidylethanolamines (LPEs), phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), sphingomyelins (SMs), ceramides (Cers), and triacylglycerols (TGs) were measured in the liver biopsies. We also determined levels of 34 BAs and 6 acyl-carnitines in the liver. Matching BAs were measured in serum. In addition, we measured the metabolites used as an index-marker of cholesterol biosynthesis (*i.e.*, lathosterol, lathosterol to cholesterol ratio (L/C), mevalonic acid phosphate), cholesterol absorption (campesterol), and BA intermediate

(3α,7α-Dihydroxy-5β-cholestanic acid, 7α-Hydroxy-3-oxo-4-cholestenoic acid) involved in cholesterol-mevalonate biosynthesis.[24]

We detected 5 PFAS in serum samples, namely perfluorohexanesulfonic acid (PFHxS), perfluorononanoic acid (PFNA), PFOA and 2 isomers of perfluorooctanesulfonic acid (PFOS) (Table S3) that were present at similar levels as previously reported in Western countries.[15,25] Additionally, we detected methylparaben and bisphenol A (BPA) and a few other compounds putatively classified as ECs. Factor analysis showed that the concentrations of metabolites and EC levels were mainly influenced by age, sex, and BMI (Fig. S1).

Environmental contaminants are associated with NAFLD

In order to study the associations between ECs and NAFLD, we developed a gradient-boosted decision tree (GBDT) model. SHapley Additive exPlanations (SHAP) values derived from these GBDT models suggested that the serum concentrations of PFAS such as PFOS, PFHxS, PFNA and PFOA were positively associated with liver fat content (R^2 = 0.79, Fig. 1A), and insulin resistance (IR) (HOMA-IR >2.5; R^2 = 0.41; Fig. 1B). PFOS, PFOA, and methylparaben were positively associated with NASH (necroinflammatory grades 1-3 [n = 21] *vs.* 0 [n = 84] (Table S1); AUC 0.85 (95% CI 0.65–0.96) (Fig. 1C), while PFOS was positively associated with hepatic fibrosis (stages 1-4 [n = 43] *vs.* 0 [n = 62] (Table S1); AUC 0.58 (95% CI 0.37–0.77) (Fig. S2). The serum levels of PFOS, PFHxS and PFNA were higher in male *vs.* female patients, while methylparaben and BPA were higher in females (Figs. 1D and 2A).

Several ECs were associated with hepatic macrosteatosis (%), HOMA-IR, stage of NASH, and fibrosis (Fig. 2A-B). The levels of PFOS were higher in females with NAFLD and IR (*vs.* without) (Fig. 2A). Meanwhile, higher levels of methylparaben (adjusted p = 0.02) were observed in males with NASH and hepatic fibrosis (*vs.* without either) (Fig. 2A).

Association of environmental contaminants with BAs and lipids are sex-specific

In order to identify EC-associated hepatic metabolites, we first clustered the molecular lipids, polar metabolites and ECs using model-based clustering (see Patients and methods), resulting in

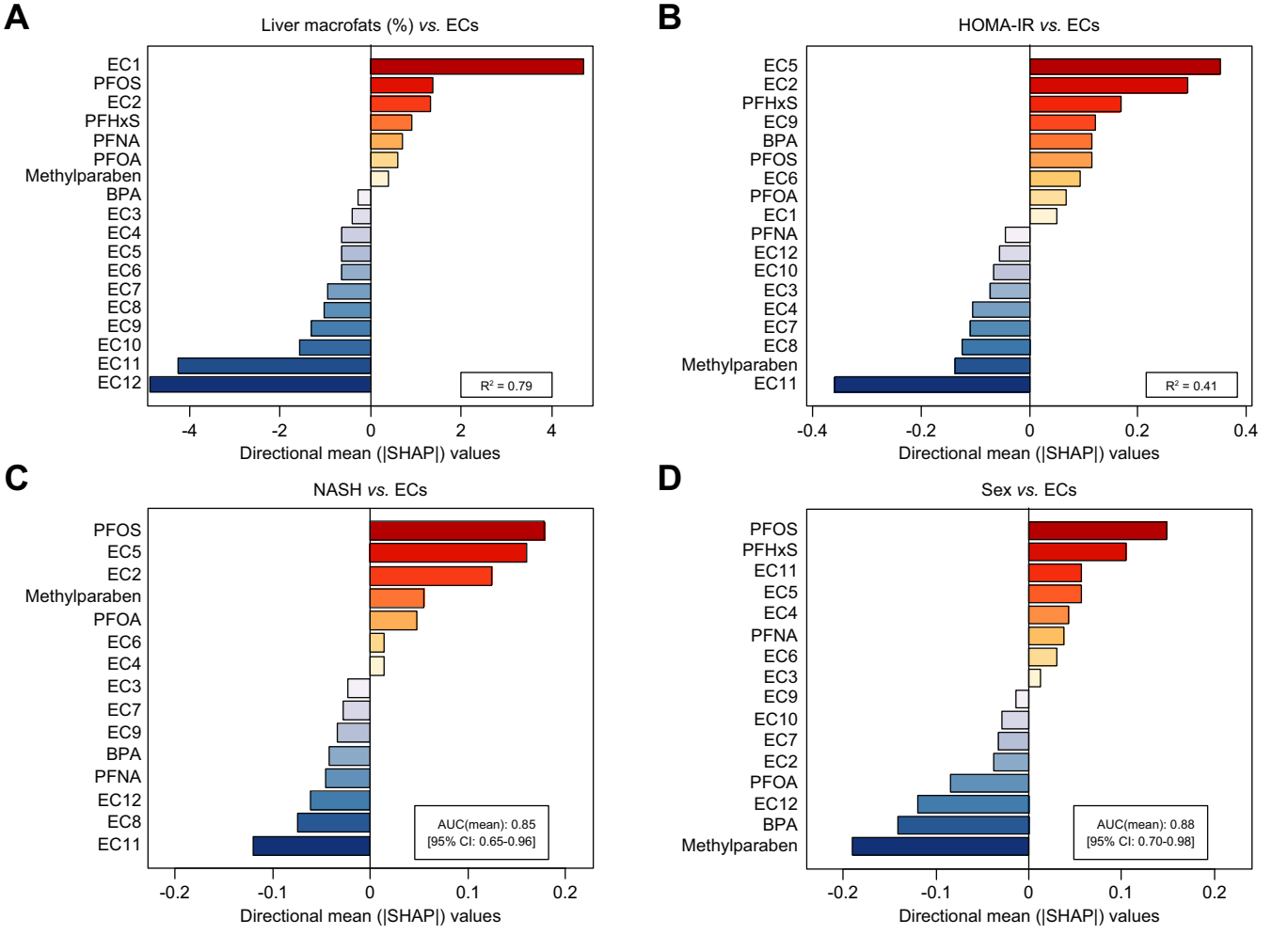

Fig. 1. Environmental contaminants associated with grading and staging of NAFLD. The directional mean |SHAP| values of ECs associated with NAFLD. (A) liver macrosteatosis (%). (B) HOMA-IR (C) NASH (grades high (1-3) *vs.* low (0)), (D) Sex. The performances of regression and classification models are given as mean R-squared (coefficient of determination), and mean area under the curve (AUCs at 95% CI). ECs, environmental contaminants; EC1, 2-(2,4-dichlorophenoxy) acetic acid; EC2, NDCA or NUDCA; EC3, 3,4-Methylenesebacic acid; EC4, C23H26O6; EC5, C15H22N2O16P2; EC6, C8H16O2; EC7, C22H30N2O5S2; EC8, xanthen-9-one; EC9, 2,6-dichloro-4-nitrophenol; EC10, 3-(4-hydroxyphenyl) propanal; EC11, C15H22O4; EC12, C21H32O2; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PFHxS, perfluorohexanesulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid.

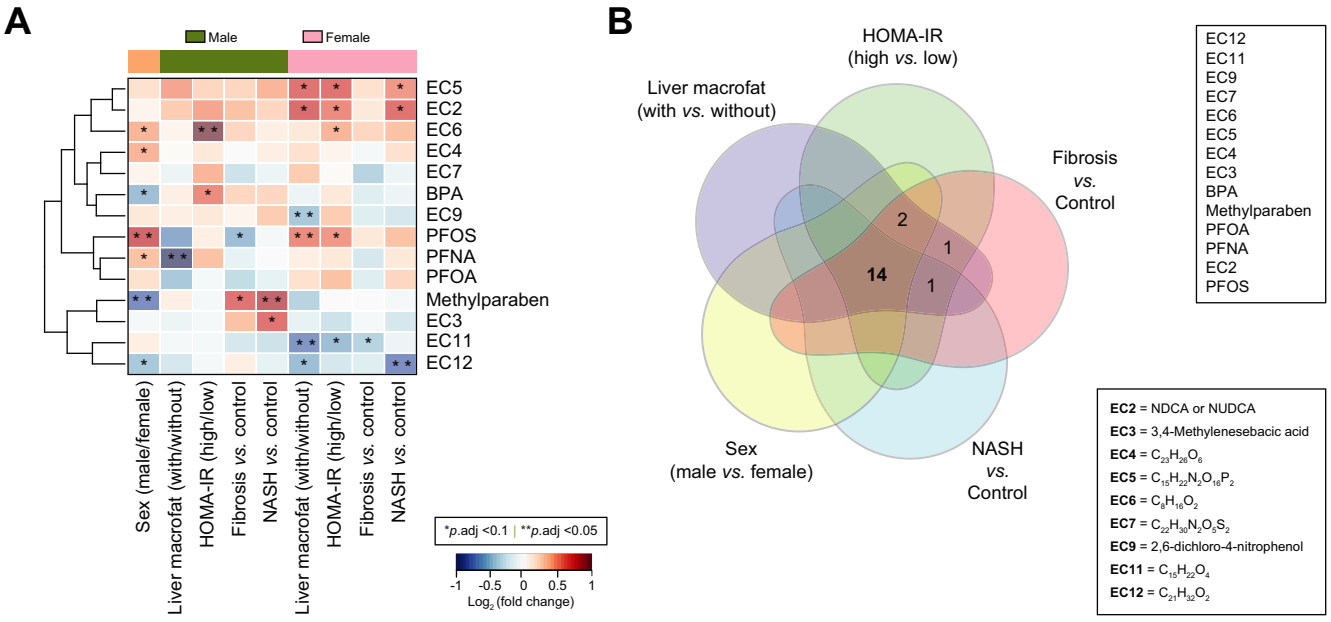

Fig. 2. Sex-specific differences in environmental contaminants linked to NAFLD. (A) Fold changes in serum levels of ECs in 2 different patient groups: liver macrosteatosis (with [n = 28/51 males/female] *vs.* without [7/19]), HOMA-IR (high (>2.5) [25/41] *vs.* low [10/29]), hepatic fibrosis (stages 1-4 (fibrosis) [16/27] *vs.* 0 (control) [19/43]), NASH (necroinflammatory grades 1-3 (NASH) [10/11] *vs.* 0 (control) [25/59]), in patients. Welch's *t* test, $^{**}p$ <0.05, $^{*}p$ <0.1, adjusted for FDR. (B) Venn diagram showing ECs that were significantly altered between the patient groups. ECs, environmental contaminants; EC2, NDCA or NUDCA; EC3, 3,4-Methylenesebacic acid; EC4, C23H26O6; EC5, C15H22N2O16P2; EC6, C8H16O2; EC7, C22H30N2O5S2; EC9, 2,6-dichloro-4-nitrophenol; EC10, 3-(4-hydroxyphenyl) propanal; EC11, C15H22O4; EC12, C21H32O2; FDR, false discovery rate; HOMA-IR, homeostatic model assessment of insulin resistance; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid.

24 distinct clusters (Table S4). These clusters contained serum environmental chemicals (2 sECC), liver BA (5 lBA), serum BA (3 sBA), liver polar metabolite (5 lPM), and liver lipid (9 lLips) clusters. Partial correlation analysis of these clusters, performed separately for males (Fig. 3A) and females (Fig. 3B), revealed markedly different correlation networks between the sexes (Fig. 3). The EC clusters in both males and females were linked to BA clusters (lBA2 and sBA1) (Fig. 3C), including positive association between sECC1 and sBA1 (CA, 7-oxo-DCA, DCA, HCA, HDCA, UDCA) and inverse association with lBA2 (GCA, GCDCA, TCA, TCDCA) (Fig. 3A-B). In males, the EC clusters were linked to lLips2 (Cers, hexosylceramides [HexCers], SMs, PCs, PEs, LPCs and LPEs) while in females they were associated with lLips5 (TGs and diacylglycerols [DGs]) and IPM3 (tricarboxylic acid cycle and its metabolites) (Table S4). In females, lLips5 was positively associated with liver fat content and NASH (Fig. 3B).

We performed a bivariate correlation analysis which linked various species of metabolites that made up the clusters (lBA2,4,5; sBA1; lLips2,5 and IPM3), with the sECC (Fig. 3 and Table S4). In females, serum concentrations of PFAS (PFNA, PFOA and PFOS) were positively associated with glyco- and tauro-conjugated primary hepatic BAs (TCA, GCDCA, TCDCA). In addition, PFOA was positively associated with multiple secondary hepatic BAs (DCA, GHCA and GUDCA) (Fig. 4). Furthermore, PFOA and PFOS were positively associated with Cers, *e.g.*, Cer(d18:0/16:0), and HexCers, *e.g.*, HexCer(d18:1/18:0), ether phospholipids, *e.g.*, O-PC(40:4), TGs and DGs (Fig. 4). Interestingly, no such association was observed in males. On the other hand, in males, levels of methylparaben and BPA were positively associated with Cers, HexCers and SMs (Fig. 4).

Next, we investigated the impact of menopause on the PFAS and metabolites (Fig. S3) using 2 age groups (<48 years and >52 years), corresponding to the menopausal transition window for most females,[26] with male patients as a control group to discern sex-specific differences. The total PFAS levels were increased (p <0.05) while levels of hepatic BAs were decreased (p = 0.02), and the levels of circulating BA remained unchanged in the post- (*vs.* pre-) menopausal female group. In males, circulating BAs were elevated (p = 0.007) while other lipids were decreased (p = 0.0002), compared to the preceding age group.

Environmental contaminants are associated with interconversion of serum BAs

We investigated the associations between ECs and specific BA ratios indicative of NAFLD and NASH, *i.e.*, ratio between primary BAs and the secondary BAs derived from them (unconjugated and conjugated [CA+DCA]/[CDCA+LCA]), the ratio increasing in NAFLD and NASH.[14] Significant (adjusted p <0.05) inverse associations between serum (CA+DCA)/(CDCA+LCA) ratio and PFNA were identified in males in both controls and patients with NASH, and also between methylparaben, and BPA in males with NASH (Fig. S4). However, in females, positive associations between the BA serum ratio and PFOA and PFOS were observed in controls, whilst female patients with NASH showed an inverse

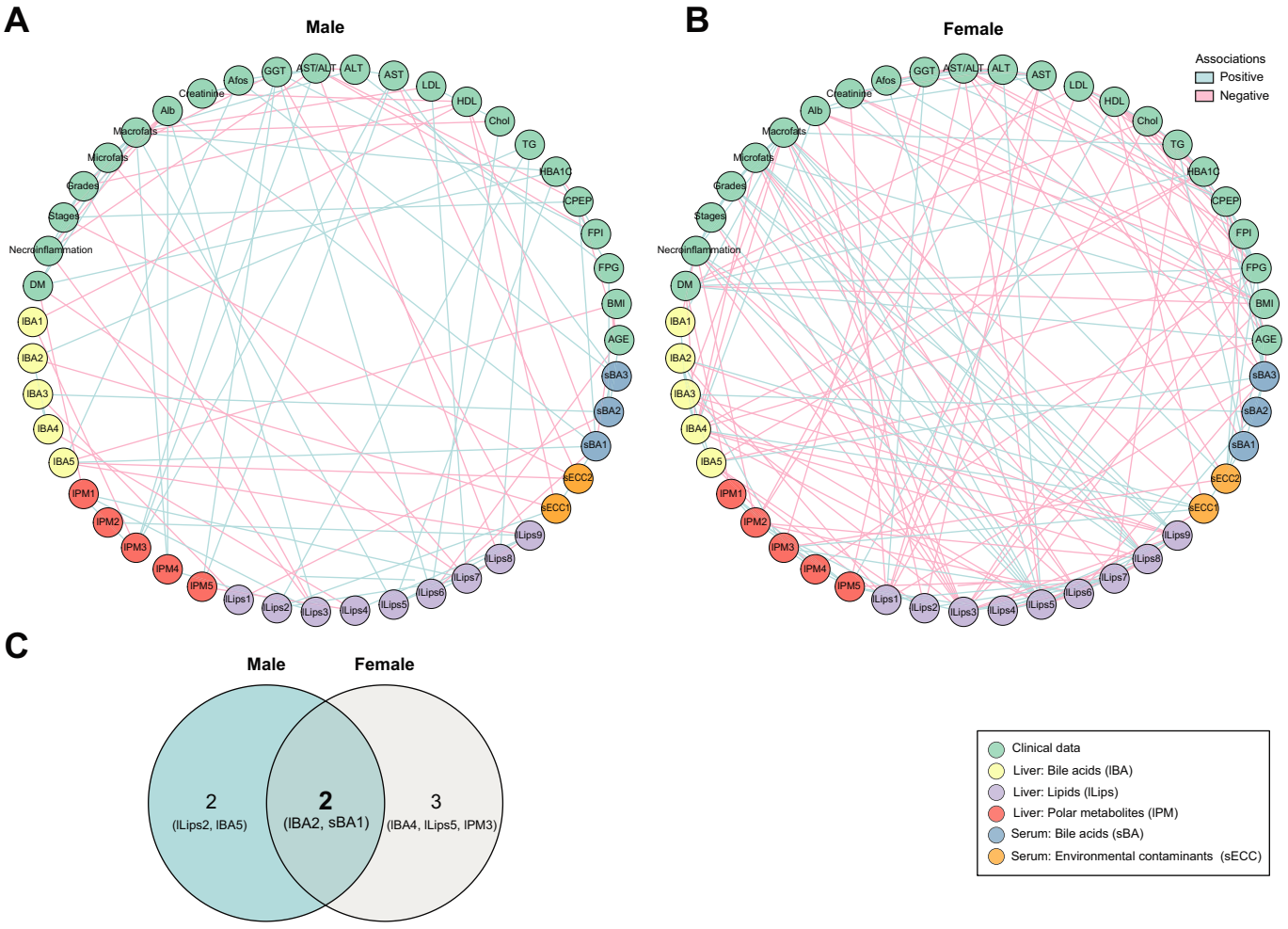

Fig. 3. Partial correlation network showing associations among the demographic, clinical data and metabolome clusters derived from liver or serum samples of males and females. (A, B) Each node represents EC (sECC) or a metabolite/lipid cluster. Associations that passed the non-rejection rates (male cut-off 0.3 and female cut-off 0.6) (Methods) are shown. Thickness of the edges are scaled by the strength of correlation coefficients. (C) Venn diagram showing metabolite clusters that are directly associated with the sECC in males and females. AFOS, plasma alkaline phosphatase; AST/ALT, plasma aspartate/alanine aminotransferase; BMR, basal metabolic rate; Chol, cholesterols; CPEP, C-Peptide; Necro, necroinflammation; DM, diabetes mellitus; FPG, fasting plasma glucose; FPI, fasting plasma insulin; GGT, gamma-glutamyltransferase; lBA, liver bile acid; lPM, liver polar metabolite; lLips, liver lipids; sBA, serum bile acid; sECC, serum environmental contaminant cluster.

association between serum BA ratio and PFHxS. Overall, the associations between PFAS and BA ratios were markedly different between controls and NASH groups (Fig. S4). In males with hepatic fibrosis, but not females, PFOA was positively associated with (CA+DCA)/(CDCA+LCA) ratio (Fig. S5). Intriguingly, a positive correlation was observed between the liver and serum levels of secondary BAs in females (Fig. S6).

Association of PFAS with the lathosterol to cholesterol (L/C) ratio; a marker of cholesterol biosynthesis

Serum L/C ratio is an index of cholesterol biosynthesis.[24] Higher L/C ratios were found in females *vs.* males both in liver and serum (Fig. S7). In males, serum L/C ratio was positively associated (adjusted p <0.05) with PFOA and negatively associated with total liver BAs, particularly CDCAs (Fig. S7). This indicates an increased level of PFOA may elevate L/C ratios, potentially impacting liver cholesterol and primary BA biosynthesis. No such associations were found in females. Instead, liver L/C ratio was positively associated (adjusted p <0.05) with PFHxS (Fig. S7). Moreover, a BA intermediate from the acidic/alternative pathway[27] was negatively associated with PFOS and PFHxS in females.

Overrepresentation of liver metabolic pathways due to chemical exposure

Pathway analysis identified multiple hepatic metabolic pathways as overrepresented in the high exposure groups (Fig. 5A-B). In both sexes, primary BA biosynthesis, glycerophospholipid metabolism, along with alanine, aspartate and glutamate metabolism were overrepresented (Fig. 5A-B). Sphingolipid metabolism pathways were overrepresented in highly exposed females (Fig. 5B). Overrepresentation of primary BA biosynthesis pathways was associated with IR in both sexes. Furthermore, the glycerophospholipid metabolism pathway was overrepresented

Fig. 4. Molecular lipids and metabolites in the human liver associated with the chemical exposure. Heatmap showing the Spearman's rank correlation coefficients between individual ECs and lipids / metabolites comprising the clusters associated with sECC in male and / or female patients, as determined by the partial correlation analysis. $^{**}p$ <0.05, $^{*}p$ <0.1, adjusted for FDR. BPA, bisphenol A; CA, cholic acid; Cer, ceramide; DCA, deoxycholic acid; DG, diacylglycerol; ECs, environmental contaminants; FDR, false discovery rate; GCA, glycocholic acid; GHCA, glycohyocholic acid; GCDCA, glycochenodeoxycholic acid; GUDCA,

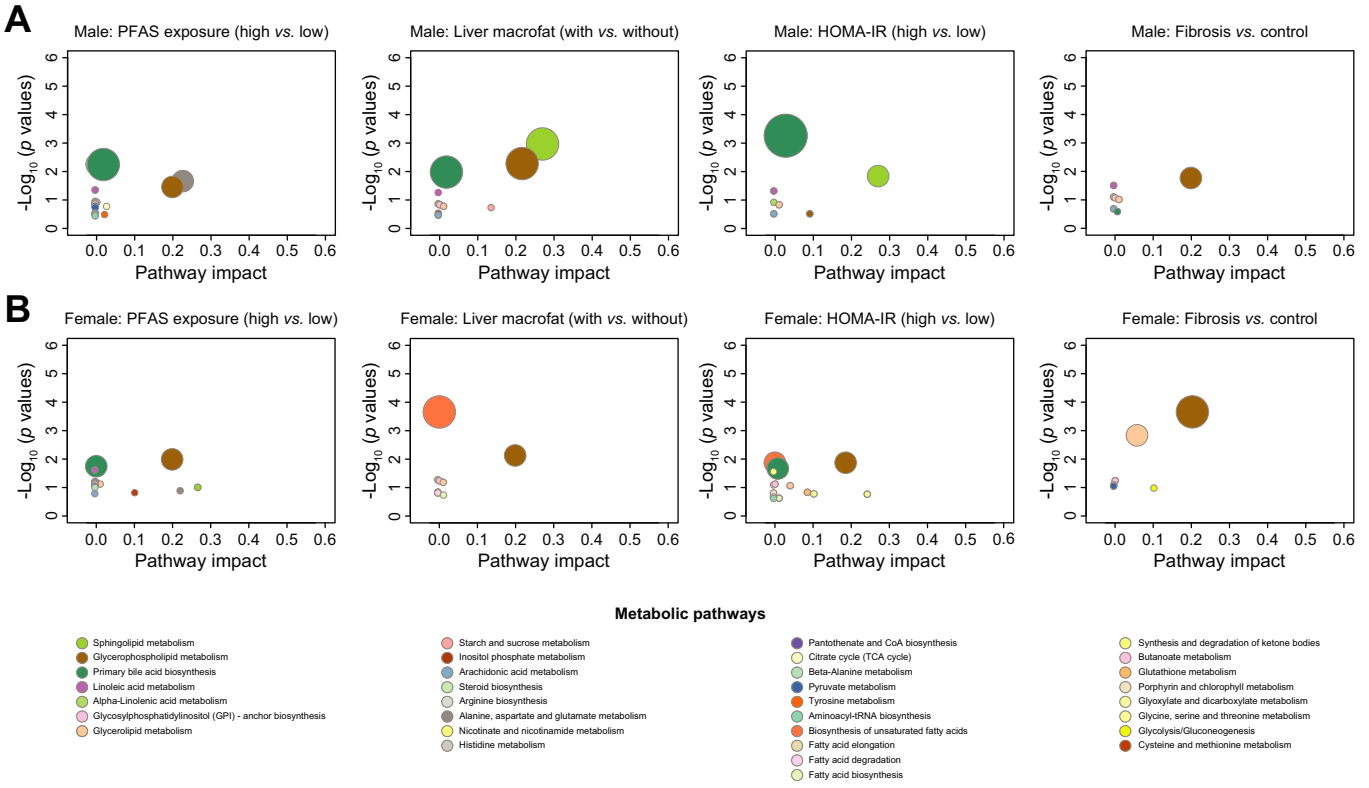

Fig. 5. Overrepresentation of metabolic pathways in the human liver impacted by the chemical exposure and/or stage of NAFLD. (A-B) Scatter showing impact scores of the liver pathways (color coded) that were overrepresented (Fisher's exact test, p <0.05) in groups: Total PFAS (PFNA, PFOS and PFOA) exposure (high *vs.* low), liver fat content (with *vs.* without), HOMA-IR (high (>0.5) *vs.* low), hepatic fibrosis (stages 1-4 (fibrosis) *vs.* 0 (control)) in male and female patients, respectively. HOMA-IR, homeostatic model assessment of insulin resistance; NAFLD, non-alcoholic fatty liver disease; PFAS, perfluorinated alkyl substances; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid.

in patients of both sexes with high macrosteatosis and increasing stages of fibrosis (Fig. 5).

Hepatic metabolic alterations in humanized PPARα mice with PFOA treatment

Here, we conducted an exposure study using a humanized PPARa (hPPARa) mouse model. We sought to confirm the aforementioned hepatic sex-specific metabolic changes as linked to chemical exposure. Of note, hPPARa mice are susceptible to hepatic steatosis.[28] Male and female hPPARa mice were provided vehicle (VH, 0.5% sucrose) or PFOA (8 μM) in their drinking water *ad libitum* for 6-7 weeks and fed a Western-type diet.

Hepatic lipids and serum BAs were measured in 2 groups (PFOA and VH). Intriguingly, several species of lipids (Cers, HexCers, SMs, LPCs, LPEs, DGs and TGs) and BAs (CA, DCA and TCA) that were positively associated with PFAS (including PFOA) exposure in the liver of female patients, were also elevated in female mice administered PFOA (*vs.* VH) (Figs. 4 and 6). Furthermore, the majority of these lipids were elevated in patients with high liver fat (Fig. 6). Thus, the animal model supported our findings, suggesting that exposure to ECs dysregulates host lipid metabolism by altering the levels of BAs, Cers, phospholipids and TGs, which, in turn, may exacerbate the risk and severity of NAFLD.

Discussion

By integrating data on biopsy-based, histologically characterized NAFLD, environmental chemical exposure and the human liver metabolome, we were able to demonstrate that circulating PFAS concentrations are associated with perturbations in key hepatic metabolic pathways previously found altered in NAFLD/NASH, particularly BA metabolism. Specifically, we identified stronger associations between the liver metabolome, chemical exposure and NAFLD-associated clinical variables in females *vs.* males. Our murine exposure study further corroborated our findings, *vis-à-vis* the association between PFAS exposure and NAFLD-associated lipid changes. Our results suggest that females may be more sensitive to chemical exposure than males, a notion that is in line with findings from other exposure studies both in animal models[20,29] and human studies.[30,31] Specifically, elevated liver enzymes were found associated with PFAS exposure only in adolescent females, with opposite results in males.[30] Also a

glycoursodeoxycholic acid; HexCer, hexosylceramide; lBA, liver bile acid; lPM, liver polar metabolite; lLips, liver lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; sBA, serum bile acid; sECC, serum EC cluster; SMs, sphingomyelin; TbMCA, tauro-β-muricholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TG, triacylglycerol.

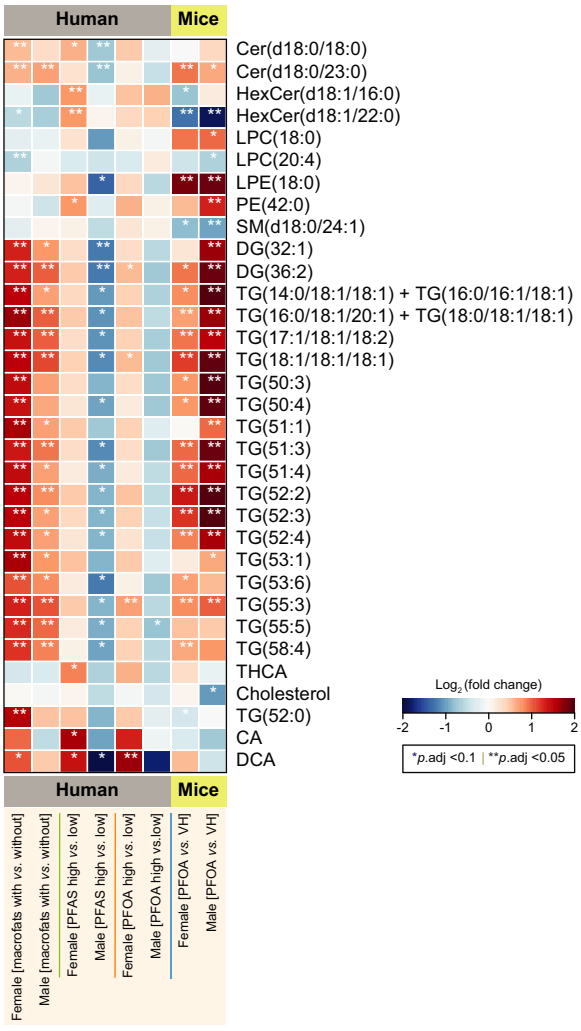

Fig. 6. Metabolites and lipids regulated in the human and hPPARa mice liver by the chemical exposure. Metabolite fold changes across different patient groups, shown separately for males and females: % of liver fat content (with *vs.* without), chemical exposure (total PFAS (PFNA, PFOS and PFOA) or PFOA levels: high *vs.* low; and PFOA *vs.* vehicle in mice. Welch's *t* test, $^{**}p$ <0.05, $^{*}p$ <0.1 (adjusted for FDR). FDR, false discovery rate; hPPAR, humanized peroxisome proliferator-activated receptor; PFAS, perfluorinated alkyl substances; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid.

recent murine study reported a higher propensity for females to develop PFAS-induced hepatic toxicity.[29]

In females, PFAS exposure was associated with increased levels of several hepatic lipids associated with NAFLD (BAs, TGs and Cers), while, surprisingly, the opposite associations were observed in males, suggesting a potentially protective effect of PFAS *via* the liver lipidome at these relatively low PFAS exposure levels. Hormesis, *i.e*, a non-monotonic dose response that results in protective effects at low doses of specific chemical exposures, has been reported in several epidemiological studies, however, the mechanism has not yet been characterized, nor identified to be sex-specific.[32,33] Indeed, in males, we observed suppression of BAs due to PFAS exposure, as indicated by the negative correlation of primary BAs and PFAS, and hardly any association between exposure and the liver lipidome. Overall, the results in males are in line with literature concerning the impact of PFAS in BA metabolism reported in animal models and *in vivo* studies, demonstrating that PFAS exposure suppresses *de novo* BA synthesis through the primary pathway *via* suppression of cholesterol 7a-hydroxylase (CYP7A1),[34] which controls the first, rate-limiting step in the formation of BAs from cholesterol. The suppression of CYP7A1 would result in downregulation of the primary BAs (CA,CDCA), as also observed in our study in males. Since part of the CDCA pool can also be synthesized *via* an alternative pathway regulated by CYP27A1[35] which is hardly affected by PFAS, the CA/CDCA ratio would be expected to decrease with higher PFAS exposure. Indeed, in males we observed decreased CA/CDCA ratios upon high PFAS exposure, indicative of suppression of the primary BA synthesis pathway, but with no impact on the alternative pathway. In females, however, the CA/CDCA ratio was not significantly associated with PFAS levels, while PFAS were positively associated with primary BAs and 2 conjugates of the secondary BA UDCA, thus indicating increased as opposed to decreased BA synthesis. This is potentially due to the reported higher expression of CYP7A1 and PPARα in the livers of females,[36] supported also by rodent models showing that females appear to be more sensitive to PFOS exposure than males.[20]

In males, positive association between PFOS and serum L/C ratio suggests that PFAS are associated with cholesterol biosynthesis. In females, the liver L/C ratio was positively associated with PFAS. It was associated with the increased levels of BAs (particularly in serum) and negatively associated with cholesterol levels both in serum and liver, suggesting an increased flux from cholesterol to BAs in females. Moreover, cholesterol absorption might be suppressed by PFAS in females, suggested by the negative association between campesterol and PFNA. This could also contribute to decreased cholesterol levels in the female liver. Together, our data indicate that PFAS exposure distinctly impacts cholesterol biosynthesis and/or absorption in males and females. However, the exact mechanisms behind these phenomena remain to be elucidated by functional studies.

The sexually dimorphic character of the liver may explain some changes observed not only in overall metabolism but also in xenobiotic metabolism, drug response and susceptibility to toxic effects.[37,38] Cholesterol metabolism is more active in females than in males,[39] with estrogen playing a key role in the regulation of hepatic lipid synthesis.[40] Sex-specific differences in glucose homeostasis have also been reported[41] while PFAS have, in *in vivo* models, been shown to have an estrogen-like activity.[42] Estrogen, and estrogenic effects, are widely known to have a protective effect against hepatic fat accumulation especially in males.[43] Alongside the biphasic, hormetic response to PFAS exposure, this represents a plausible explanation for the protective effects of the PFAS exposure we observed in the males of our study.

In females, serum BAs positively associated with PFAS exposure were also linked with glucose metabolism, indicating that PFAS may disrupt the interplay of BA and glucose metabolism. In males, we did not observe associations between ECs and glucose metabolism. In females, PFAS and several BAs in serum were positively associated with HOMA-IR, and importantly, the 12a-hydroxylated BAs (CA, DCA, and their conjugates) were associated with both FPG and HOMA-IR. Increased 12a-hydroxylated BAs have been reported in IR[44] with increased primary and total BAs reflecting IR in patients with NASH,[45] which is in agreement

with our findings in females. The serum ratio of specific BAs ([CA + DCA]/[CDCA + LCA]), used as a surrogate for changes in BA biosynthesis, was associated with several ECs in females, further indicating disturbances in BA metabolism. This increased BA ratio has also been reported in patients with IR and hepatic steatosis,[14] which may explain the link we observed between PFAS and glucose metabolism. Interestingly, this specific BA ratio was different in both females and males with low or high NASH/fibrosis scores. In more severe stages of liver disease, the PFAS-BA associations were similar for both sexes. Pathway analysis further showed that glycerophospholipid metabolism was over-represented in the liver of both males and females in the advanced stages of NAFLD, suggesting that these metabolic pathways could be a common denominator contributing to the increased risk of NAFLD by chemical exposure.

The changes in BAs were more likely to be linked with PFAS exposure than NAFLD *per se*, as we did not observe any significant increases in hepatic BAs in NAFLD, except for weak associations (elevated LCA, downregulated TMCA) in females. While elevated levels of BAs in NAFLD have been reported,[46] some of the results may be linked with obesity rather than NAFLD/NASH due to a significant difference in the BMI of controls *vs.* patients with NAFLD in many studies[45] and as elevated BA concentrations have been reported in obese patients without NAFLD.[47] In our study, all patients, including those with normal liver fat, were obese (BMI >30), thus allowing us to control for the effect of BMI.

The comparison of our human data with those from the mouse model showed that in females, similar lipid changes were triggered in mice (male and female) due to PFOA exposure. However, in human females, the changes did not always reach statistical significance, most likely due to lower exposure levels than used in the mouse model or due to the differences in estrogen levels in human females due to their age distribution (29–60 years). Importantly, the PFOA-induced changes in mice showed a strong association with those lipids that, in humans, were associated with liver fat in both males and females, and with glucose homeostasis in females, thus supporting the notion that PFAS exposure may contribute to the development of NAFLD. Our results also showed that glucose homeostasis is linked with PFAS exposure in females but not in males, a finding that demands further investigation, along with addressing the effect of menopause on glucose homeostasis.

A particular strength of our study is that it is based on a comprehensive metabolic characterization of both liver and serum in a well-characterized human cohort, including assessment of NAFLD stages and severity, based on liver biopsy, with key results further verified in a murine exposure study. There are also some limitations, including that we did not have data on the hormonal status of the females, or data regarding hormone replacement therapy in postmenopausal females. Moreover, due to the cross-sectional study design, the temporal relationship between PFAS and lipids may not be fully representable. Overall, our results suggest that females may be more sensitive to the harmful impacts of PFAS, even at lower PFAS exposure levels. The results also suggest that the changes reported in lipid metabolism subsequent to PFAS exposure may be secondary to the interplay of PFAS and BAs.

Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BAs, bile acids; BPA, bisphenol A; CA, cholic acid; Cers, ceramides; CYP7A1, cholesterol 7a-hydroxylase; DCA, deoxycholic acid; DG, diacylglycerol; EC, environmental contaminants; EDCs, endocrine-disrupting chemicals; GBDT, gradient-boosted decision tree; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; HexCers, hexosylceramides; HOMA-IR, homeostatic model assessment of insulin resistance; hPPARa, humanized PPARa; IR, insulin resistance; L/C, lathosterol to cholesterol ratio; lBA, liver bile acid; LCA, lithocholic acid; lLips, liver lipids; LPCs, lysophosphatidylcholines; LPEs, lysophosphatidylethanolamines; lPM, liver polar metabolite; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PCs, phosphatidylcholines; PEs, phosphatidylethanolamines; PFAS, perfluorinated alkyl substances; PFHxS, perfluorohexanesulfonic acid; PFNA, perfluorononanoic acid; PFOA, perfluorooctanoic acid; PFOS, perfluorooctanesulfonic acid; PPAR, peroxisome proliferator-activated receptor; sECC, serum EC cluster; sBA, serum bile acid; SMs, sphingomyelins; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; TG, triacylglycerol.

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Conflict of interest

The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceptualization and design of study (MO, HYJ, TH), clinical study (AJ, JA, coordinated by HYJ), murine study (JJS, TFW), metabolomics analysis (OR, SJ, supervised by TH), data analysis and interpretation (PS, with critical input from SQ, PKL, AM, HYJ, TH, supervised by MO). Manuscript preparation (PS, MO, TH), Critical review and editing (all authors).

Data availability statement

Data from the clinical study are available upon request and an appropriate institutional collaboration agreement. These data are not available to access in a repository owing to concern that the identity of patients might be revealed inadvertently. Data from the murine study are available from the Metabolomics Workbench (https://www.metabolomicsworkbench.org/) as DOI https://doi.org/10.21228/M89D7G.

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Supplementary data

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Author names in bold designate shared co-first authorship

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