In utero exposures to perfluoroalkyl substances and the human fetal liver metabolome in Scotland: a cross-sectional study

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Summary

Background Perfluoroalkyl and polyfluoroalkyl substances are classed as endocrine disrupting compounds but continue to be used in many products such as firefighting foams, flame retardants, utensil coatings, and waterproofing of food packaging. Perfluoroalkyl exposure aberrantly modulates lipid, metabolite, and bile acid levels, increasing susceptibility to onset and severity of metabolic diseases, such as diabetes and metabolic dysfunction-associated steatotic liver disease. To date, most studies in humans have focused on perfluoroalkyl-exposure effects in adults. In this study we aimed to show if perfluoroalkyls are present in the human fetal liver and if they have metabolic consequences for the human fetus.

Methods In this cross-sectional study, human fetal livers from elective termination of pregnancies at the Aberdeen Pregnancy Counselling Service, Aberdeen, UK, were analysed by both targeted (bile acids and perfluoroalkyl substances) and combined targeted and untargeted (lipids and polar metabolites) mass spectrometry based metabolomic analyses, as well as with RNA-Seq. Only fetuses from normally progressing pregnancies (determined at ultrasound scan before termination), terminated for non-medical reasons, from women older than 16 years, fluent in English, and between 11 and 21 weeks of gestation were collected. Women exhibiting considerable emotional distress or whose fetuses had anomalies identified at ultrasound scan were excluded. Stringent bioinformatic and statistical methods such as partial correlation network analysis, linear regression, and pathway analysis were applied to this data to investigate the association of perfluoroalkyl exposure with hepatic metabolic pathways.

Findings Fetuses included in this study were collected between Dec 2, 2004, and Oct 27, 2014. 78 fetuses were included in the study: all 78 fetuses were included in the metabolomics analysis (40 female and 38 male) and 57 fetuses were included in the RNA-Seq analysis (28 female and 29 male). Metabolites associated with perfluoroalkyl were identified in the fetal liver and these varied with gestational age. Conjugated bile acids were markedly positively associated with fetal age. 23 amino acids, fatty acids, and sugar derivatives in fetal livers were inversely associated with perfluoroalkyl exposure, and the bile acid glycolithocholic acid was markedly positively associated with all quantified perfluoroalkyl. Furthermore, 7α-hydroxy-4-cholesten-3-one, a marker of bile acid synthesis rate, was strongly positively associated with perfluoroalkyl levels and was detectable as early as gestational week 12.

Interpretation Our study shows direct evidence for the in utero effects of perfluoroalkyl exposure on specific key hepatic products. Our results provide evidence that perfluoroalkyl exposure, with potential future consequences, manifests in the human fetus as early as the first trimester of gestation. Furthermore, the profiles of metabolic changes resemble those observed in perinatal perfluoroalkyl exposures. Such exposures are already linked with susceptibility, initiation, progression, and exacerbation of a wide range of metabolic diseases.

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Introduction

Metabolic diseases caused by environmental exposures can begin in utero, and as early as the first trimester of pregnancy. This impact of early-life exposure on metabolic disease risk is termed early metabolic programming of adult health.[1] Prenatal development determines the foundation for overall metabolism in fetal tissues, and altered development can substantially affect health in later life. The liver is central to metabolic health in adults and liver development begins with hepatocyte differentiation during the eighth week of gestation.[2] Published data on the human fetal liver metabolome are limited.

The developing liver is highly sensitive to environmental triggers, with overwhelming evidence supporting the concept that exposure to xenobiotic compounds

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Research in context

Evidence before this study

We searched Scopus and PubMed using the terms "perfluorinated alkyl substances", "PFAS", "PFOA", "PFOS", "PFHxS" and "metabolomics" or "lipidomics" and "liver", "fetal", "hepatocytes" for articles in English from database inception until Oct 15, 2023. Biomonitoring studies have shown that perfluorinated alkyl substances, a known class of environmental pollutants and endocrine disrupting compounds, are detected in almost all humans, and that these compounds are efficiently transported to the fetus through the placenta. Epidemiological studies have reported that prenatal exposure to perfluoroalkyl is associated with multiple adverse health impacts later in life, suggesting that exposure causes changes in developmental programming.

Added value of this study

To our knowledge this is the first study to both measure perfluoroalkyl in the human fetal liver and carry out comprehensive metabolic profiling of the tissue. The study provides evidence that the levels of perfluoroalkyl detected in human fetal livers are associated with alteration of fetal liver metabolome.

Implications of all the available evidence

Identification of metabolic perturbations in the human fetus associated with perfluoroalkyl exposure shows that environmental exposure and its potential harmful effects start in utero. These effects of perfluoroalkyl in the fetus, particularly with respect to lipid and bile acid metabolism, might be responsible for reported adverse health effects during early life, and is likely to have a role in the developmental origins of health and disease.

affects fetal development, increasing risk profiles for disease later in life.[3] Moreover, genetic and environmental perturbations can lead to maladaptive metabolic responses and serious adverse consequences for the fetus, which has incomplete organ development with limited resistance and detoxification capabilities. Further, environmental endocrine disrupting chemicals can promote metabolic changes that result in fatty liver.[4]

One class of endocrine disrupting chemicals to which humans are widely exposed are perfluoroalkyl substances. These compounds are used in many industrial and commercial products, persist in the environment, and bioaccumulate. Perfluoroalkyl exposure is associated with multiple adverse health outcomes across all life stages, including liver damage, increased serum cholesterol levels, decreased immune response, increased risk of thyroid disease, decreased fertility, pregnancy-induced hypertension, pre-eclampsia, lower birthweight, and testicular and kidney cancer.[5–8] This disease risk burden is reflected in the perfluoroalkyl-attributable disease costs that have been evaluated to be around €52–84 billion for all European Economic Area countries[9] and in the USA the costs were estimated to be around US$5·52 billion, with sensitivity analyses revealing costs reaching $62·6 billion.[10] Importantly, the largest economic contributor to the main estimate of disease costs in the USA attributable to perfluoroalkyl was childhood obesity.[10]

Perfluoroalkyls can be transported from maternal blood to cord blood through the placenta and it has been shown that perfluoroalkyls in the fetus are enriched in the liver and lungs.[11] However, mechanisms underlying effects of prenatal perfluoroalkyl exposure on the fetal liver remain unclear. Animal studies show that even in utero, perfluoroalkyl exposure might cause elevated liver enzymes, liver enlargement, and hepatic steatosis, with increased oxidative stress, and immunotoxic effects.[12] Epidemiological studies in adults also consistently report similar impacts.[7] Developmental exposure to perfluorooctanoic acid, a common perfluoroalkyl, has also been linked with altered weight gain, decreased serum leptin, and reduced insulin sensitivity later in life.[13] Moreover, perfluoroalkyl exposure affects peroxisome proliferator-activated receptor-alpha (PPARα) and peroxisome proliferator-activated receptor-gamma as well as hepatocyte nuclear factor 4 (HNF4) activities, which have important roles in transcriptional regulation of lipid and amino acid metabolism.[14] A 2015 study using *PPARα*$^{-/-}$ mice suggests that perfluorooctanoic acid can induce liver toxicity, via PPARα-independent mechanisms,[15] and that these effects might be mediated in utero.

Perfluoroalkyls affect multiple metabolic pathways, including lipid, energy, amino acid, cholesterol, and bile acid metabolism,[8] the results suggesting that the liver, as the main metabolic organ responsible for lipid and steroid metabolism, has a key role in mediating these effects. Results from our 2020 and 2022 studies suggest that many of these perfluoroalkyl-associated changes might be mediated by the bile acids,[16,17] which are important regulators of hepatic lipid and glucose metabolism. Importantly, unlike in adults, fetal bile acids are synthesised in the liver mainly via an alternative pathway mediated by cholesterol 27α-hydroxylase (CYP27A). Given liver immaturity, enterohepatic circulation of bile acids is thought to be absent in the fetus and the metabolic processes of bile acids undeveloped. However, several non-typical secondary bile acids (1β-hydroxylated and ketonic bile acids) have been detected in the fetus, distinct from the corresponding maternal profile, suggesting that they are indeed synthesised in the fetal liver.[18] Overall, primary bile acids predominate in the fetal bile acid pool and are transferred to the mother across the placenta and converted into secondary bile acids by maternal intestinal microflora. Secondary bile acids in maternal circulation can be transported back to the fetus through the placenta.

Therefore, fetal blood bile acid concentrations are maintained by fetal–placental–maternal bile acid circulation.

In this study our objective was to examine the associations between perfluoroalkyl exposure and the human fetal liver metabolome and transcriptome and identify any variations with stage of gestation or fetal sex. We have applied three mass spectrometry-based methods to measure the metabolome and to quantitatively assess the concentrations of perfluoroalkyls and bile acids in the fetal liver. Data from these measurements have also been supported by transcriptome analysis of the same tissues.

Methods

Study design and participants

This was a cross-sectional study with retrospective construction of analytical cohorts for metabolic analysis of human fetal livers from elective termination of pregnancies. Women seeking elective terminations of pregnancy were recruited with full written, informed consent by nurses working independently of the study at Aberdeen Pregnancy Counselling Service, Aberdeen, UK. In this study the terms woman, women, maternal, or mother refer to people having a termination of pregnancy. Data were not collected on gender identity for this research. Fetuses included in this study were collected between Dec 2, 2004, and Oct 27, 2014. Only fetuses from normally progressing pregnancies (determined at ultrasound scan before termination), terminated for non-medical reasons, from women older than 16 years, fluent in English, and between 11 and 21 weeks of gestation were collected. Women exhibiting considerable emotional distress or whose fetuses had anomalies identified at ultrasound scan were excluded. Fetuses were weighed, sexed, and the crown-rump length recorded. Fetal sex was determined by fetal morphology and validated using at least one of the following: gonadal morphology, presence of Y chromosome (male specific), and checked by high expression levels of *XIST* (female marker) in RNAseq data.

Collection of fetal material used in this study was approved by the National Health Service (NHS) Grampian Research Ethics Committees (REC 04/S0802/21) and conducted according to the guidelines laid down in the Declaration of Helsinki. STROBE compliance methods and checklist are in the appendix (pp 2–4).

Mass spectrometric analyses

A targeted, quantitative method was used for the analysis of fetal liver tissue perfluoroalkyls and bile acids (including 7α-hydroxy-4-cholesten-3-one [C4]) as described previously,[19] and two combined target-non-target methods were used for the analysis of semipolar metabolites and other pollutants as well as for lipidomics. The analyses were performed by ultra high-performance liquid chromatography combined with either triple quadrupole mass spectrometry (targeted analyses, in both positive and negative ionisation modes) or quadrupole time-of-flight mass spectrometry (combined targeted and untargeted analyses, in both positive and negative ionisation mode). The liver samples were weighed, and phosphate-buffered saline was added and the samples were homogenised. Separate extraction methods were used for lipids and for polar and semipolar metabolites and peralkyl substances. For lipidomics, the samples were extracted using a modified version of the previously described Folch procedure.[16] Processing of the raw mass spectrometry data was performed using the open-source software Mzmine 2.18.[20] Full details of mass spectrometric analyses, data processing, and quality control are in the appendix (pp 5–8, 15–21).

Transcriptomics analyses

RNA was extracted from 80 fetal livers using the AllPrep DNA/RNA/Protein mini kit (Qiagen, Manchester, UK) as per manufacturer's instructions and prepared for sequencing using the Illumina TruSeq Stranded mRNA library prep kit (Illumina, Cambridge, UK). mRNA was converted to cDNA as per the library preparation kit and ctDNA sequencing was done at the Centre for Genome Enabled Biology and Medicine at the University of Aberdeen using an Illumina NextSeq 500 platform (Illumina, Cambridge, UK). Raw data were quality filtered with TrimGalore! (version 0.4.0), aligned to human reference genome GRCh38 using Hisat2 (version 2.0.1), and reads at genes were quantified using featureCounts (part of the sub read version 5.0.1 package). Normalised read counts were produced using edgeR (version 3.12.1).

For more on **TrimGalore!** see https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/

For more on **Hisat2** see https://daehwankimlab.github.io/hisat2/

For more on **featureCounts** see https://subread.sourceforge.net/featureCounts.html

For more on **edgeR** see https://bioconductor.org/packages/release/bioc/html/edgeR.html

Statistical analysis

Data analysis was carried out in the R statistical programming environment (version 4.2.2). Preprocessing of metabolomics data involved replacement of zero values with imputed half-minimums for their respective feature, $\log_2$ transformation, and autoscaling. Clustering of the polar metabolites dataset was carried out using Gaussian finite mixture modelling. A representative value for each sample's exposure to all measured individual perfluoroalkyls was calculated by weighted quantile sum (WQS) regression using the wqs() R package, version 0.0.1. Spearman correlation was calculated pairwise between all molecular and clinical variables (corrplot R package version 0.84). Associations likely to be spurious were rejected using non-rejection rate calculation and a network of remaining associations projected from the non-rejection rate-filtered pairwise correlation matrix. Metabolic pathway enrichment analyses and linear regression modelling analysis were carried out using the MetaboAnalyst tool (version 5.0).[21] Full methods and parameters are given in the appendix (pp 8–9).

See **Online** for appendix

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

78 fetuses were included in the study: all 78 fetuses were included in the metabolomics analysis (40 female and 38 male) and 57 fetuses were included in the RNA-Seq analysis (28 female and 29 male; table).

Using four different analytical platforms (two for targeted methods and two for combined targeted and untargeted methods), we identified 196 lipids and 64 free fatty acids, bile acids, amino acids, and other polar metabolites, perfluoroalkyls, and other environmental chemicals in 78 liver samples (appendix pp 15–21). The lipid composition was dominated by phosphatidylcholines, phosphatidylethanolamines, and triacylglycerols as well as several polyunsaturated free fatty acids. The major fetal liver bile acids were taurine conjugated primary bile acids taurochenodeoxycholic acid and taurocholic acid, followed by glycine conjugated glycochenodeoxycholic acid and glycocholic acid. Overall, most bile acids detected were conjugated, and the proportion of unconjugated bile acids was less than 1·5% of the total bile acid pool. Taurine-conjugated bile acids dominated the bile acid pool (>73%). In addition to the primary bile acids, we detected multiple secondary bile acids as well as bile acids classified both as primary and secondary—ie, these bile acids can be produced by the liver and also formed from primary bile acids by gut microbiota, or alternatively, could be transferred from the maternal circulation. 13 perfluoroalkyls were detected in the fetal liver samples although not all were detected in less than 20% of the samples. Five perfluoroalkyls were detected in most of the samples (>80%), namely perfluorooctanesulfonic acid, perfluorooctanoic acid, perfluorohexanesulphonic acid, perfluorohexanoic acid, and perfluoroundecanoic acid, and a singular value for overall exposure, calculated by WQS regression, was included in further data analyses.

Given the large size of the polar metabolite dataset, including identified (n=47) and unknown (n=6675) metabolites, the dataset was grouped into 15 clusters (appendix pp 11, 22). Lipids were amalgamated into the following lipid classes: cholesterol esters, ceramides, lysophosphatidylcholines,lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylethanolamines, phosphatidylinositols, sphingomyelins, triacylglycerols-containing monounsaturated fatty acids, triacylglycerols containing polysaturated fatty acids, and triacylglycerols containing saturated fatty acids.

We investigated, and subsequently adjusted for, the effect of possible confounding factors on our results using a generalised linear model (appendix p 9), namely, fetal age, fetal sex, maternal age, maternal BMI, maternal smoking status, and number of previous pregnancies (including livebirths and previous terminations). Of these, fetal age and fetal sex had the most effect on metabolome and perfluoroalkyl concentrations, while the other factors did not show significant effect on either metabolome or perfluoroalkyl concentrations (appendix p 12). Sampling year or storage duration did not show any significant association with either metabolome or perfluoroalkyl concentrations (linear regression, adjusted with gestational age and maternal BMI).

To investigate associations between metabolites, pollutants, and clinical variables, correlation network analysis was carried out (figure 1). When considering all robust (non-rejection rate pruned) associations, as shown in the appendix (p 13), most lipid classes positively associated primarily amongst themselves, although an association plane exists between cholesterol esters, lysophosphatidylcholines, and lysophosphatidylethanolamines and the metabolites or perfluoroalkyl region, as well as through tauro-α-muricholic acid (TαMCA) and tauro-β-muricholic acid (TβMCA). Similarly, bile acids mostly positively associated among themselves.

With respect to the bile acids, TαMCA or TβMCA and tauro-ω-muricholic acid associated with lipid classes, metabolites, and WQS-weighted perfluoroalkyl (figure 1). A notable observation is the highly interconnected role ascribed to glycolithocholic acid, which had strong

	Fetal sex		p value*
	Female	Male	
Metabolomics cohort			
Number of fetuses in cohort	40	38	..
Weeks of gestation	14·5 (0·4)	15·0 (0·4)	0·32
Fetal weight, g	74·3 (11·0)	87·1 (12·6)	0·45
Fetal crown to rump length, mm	96·4 (4·5)	102 (4·5)	0·34
Maternal age, years	23·5 (0·9)	25·8 (1·1)	0·10
Maternal BMI, kg/m²	24·7 (0·7)	25·0 (0·8)	0·77
Perfluoroalkyl substances in the fetal liver, pg/mg			
Perfluoropentanoic acid	0·128 (0·069–0·384)	0·127 (<LOD–0·343)	0·20
Perfluorohexanoic acid	0·039 (<LOD–0·148)	0·042 (<LOD–0·085)	0·64
Perfluoroheptanoic acid	<LOD (<LOD–0·033)	<LOD (<LOD–0·048)	0·80
Perfluorohexanesulphonic acid	0·051 (<LOD–0·200)	0·0623 (0·010–0·223)	0·47
Perfluorooctanoic acid	0·114 (0·054–0·320)	0·108 (0·063–0·327)	0·84
Perfluorononanoic acid	<LOD (<LOD–0·111)	<LOD (<LOD–0·214)	0·97
Perfluorooctanesulfonic acid	0·543 (0·231- 4·88)	0·600 (0·211–1·35)	0·79
Perfluorodecanoic acid	0·023 (<LOD–0·108)	0·020 (<LOD–0·053)	0·59
Perfluoroundecanoic acid	0·052 (0·019–0·164)	0·048 (0·028–0·100)	0·59
Perfluorotridecanoic acid	<LOD (<LOD–0·116)	<LOD (<LOD–0·080)	0·20
Perfluorotetradecanoic acid	0·039 (<LOD–0·116)	0·040 (<LOD–0·070)	0·33
Perfluorohexadecanoic acid	0·271 (0·153–0·820)	0·287 (0·163–0·593)	0·78
RNA sequencing cohort			
Number of fetuses in cohort	28	29	..
Weeks of gestation	15·3 (0·4)	15·0 (0·4)	0·63
Fetal weight, g	95·9 (14·5)	85·5 (14·4)	0·69
Fetal crown to rump length, mm	105·0 (4·9)	103·0 (5·4)	0·86
Maternal age, years	22·6 (1·0)	26·6 (1·0)	0·0053
Maternal BMI, kg/m²	25·4 (1·0)	25·2 (0·9)	0·99

Data are n, mean (SEM), or median (minimum–maximum). The RNA sequencing cohort overlaps with the metabolomics cohort. LOD=limit of detection. *Student's *t*-test.

Table: **Fetal cohort demographic data (N=78)**

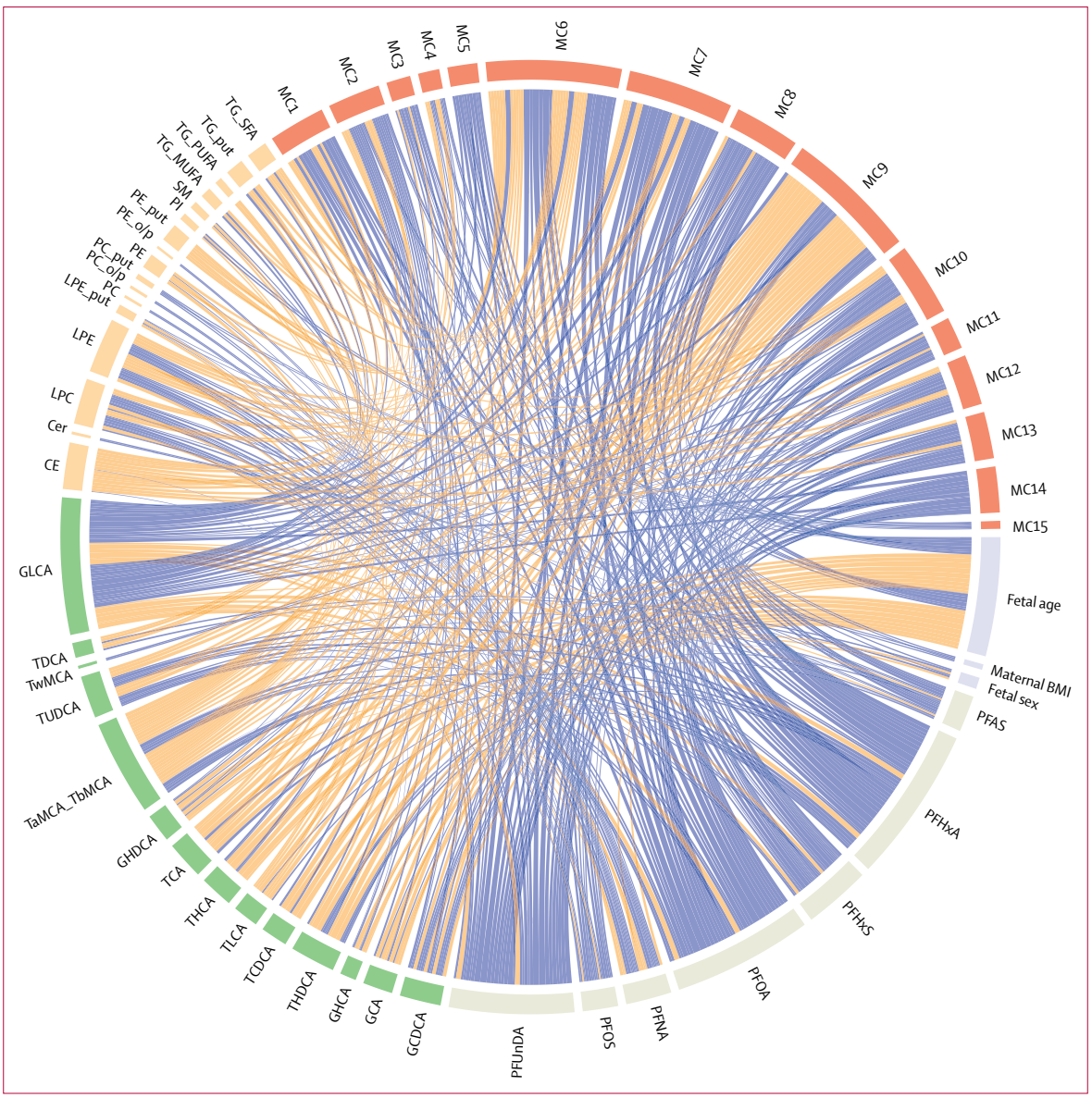


Figure 1: **Circular network projection of all study MC lipid classes, perfluoroalkyls, bile acids, and demographic data (fetal sex and BMI of the pregnant woman)**

The network is pruned to include only those associations deemed to be non-spurious as per filtering of all pairwise Spearman correlations which passed the non-rejection rate threshold of 0·15. Intra-dataset connections (eg, between perfluoroalkyl and other perfluoroalkyl, lipid classes, and other lipid classes) are removed for clarity to show interactions between different classes of variables. Perfluoroalkyl here refers to the weighted quantile sum regression-calculated value for overall exposure to perfluoroalkyl. Edge colour denotes the directionality of the association whereby blue is a negative association and orange is a positive association. Edge thickness is a function of association strength, with thicker edges denoting stronger associations by Spearman correlation. Colours of segments next to feature names group features into their overarching types: red is metabolite clusters, grey is perfluoroalkyl substances, light blue is demographic data, green is bile acids, and orange is lipids. CE=cholesterol esters. CeR=ceramides. GCA=glycocholic acid. GCDCA=glycochenodeoxycholic acid. GHCA=glycohyocholic acid. GHDCA=glycohyodeoxycholic acid. GLCA=glycolithocholic acid. LPC=lysophosphatidyl choline. LPE= Lysophosphatidylethanolamine. LPE_put=Lysophosphatidyletha nolamine putative. MC=metabolite cluster. PC_o/p=alkylether phosphatidyl choline. PC_put=phosphatidyl choline putative. PC=phosphatidyl choline. PE_o/p=alkylether phosphatidylethanolamine. PE_put=phosphatidylethanolamine putative. PE=phosphatidylethanolamine. PFAS=perfluoroalkyl substances. PFBA=perfluorobutanoic acid. PFHxA=perfluoro hexanoic acid. PFHxS=perfluorohexanesulfonic acid. PFOA=perfluorooctanoic acid. PFOS=perfluorooctanesulfonic acid. PFUnDA=perfluoroundecanoic acid. PI=phosphatidylinositol. SM=sphingomyelin. TaMCA_TbMCA=tauro-α-muricholic acid tauro-β-muricholic acid. TCA=taurocholic acid. TCDCA=taurochenodeoxycholic acid. TDCA=taurodeoxycholic acid. TG_MUFA=triacylglycerol monounsaturated fatty acids. TG_PUFA=triacylglycerol polyunsaturated fatty acids. TG_put=triacylglycerol putative. TG_SFA=triacylglycerol saturated fatty acids. THCA=taurohyocholic acid. THDCA=taurohyodeoxycholic acid. TLCA=taurolithocholic acid. TUDCA=tauroursocholic acid. TwMCA=tauro-ω-muricholic acid.

inverse associations with several metabolite clusters, and strong positive associations with various perfluoroalkyls. This pattern was not observed for any other bile acid. Of the perfluoroalkyls, perfluorohexanoic acid was the most interconnected by associations with the metabolite clusters and various bile acids.

Fetal age (gestation weeks) was positively associated with mainly conjugated bile acids and inversely associated with eight metabolite clusters (figure 1; figure 2A, B). There were positive associations between fetal age and four cholesterol esters, ten phosphatidylcholines, two phosphatidylethanolaminess, one phosphatidylinositol, two sphingomyelins, and six triacylglycerols; and fetal age associated negatively with 11 phosphatidylcholines, one phosphatidylinositol, ten sphingomyelins, and 12 triacylglycerols (appendix pp 23–24). 14 identified polar metabolites were significantly ($p<0·05$) associated with age (appendix p 25), including free fatty acids inversely associating with fetal age. Only one metabolite was affected by maternal BMI and five by fetal sex; one metabolite affected by maternal BMI and two metabolites affected by fetal sex overlapped with metabolites associating with fetal age (appendix p 26), indicating that fetal age effects on the fetal hepatic metabolome was not confounded by maternal BMI or fetal sex.

Metabolic pathway enrichment analysis using the full list of polar metabolites' p values and correlation values (as *t*-scores) was also performed (appendix p 27). Most enriched pathways that associated with fetal age were attributed to lipid metabolism and inflammatory pathways.

Following correction for fetal age, maternal BMI, and fetal sex using a general linear model, the potential relationships between perfluoroalkyl (including WQS-weighted perfluoroalkyl score) and all lipids, polar metabolites, and bile acids was assessed (figure 3). Three bile acids (tauroursodeoxycholic acid, TαMCA, or TβMCA) were negatively related with fetal perfluoroalkyl (figure 3A, B). In contrast, the known liver-toxicant glycolithocholic acid was strongly and positively related with all perfluoroalkyl except perfluorooctanesulfonic acid (and WQS-weighted perfluoroalkyl exposure, which is dominated by perfluorooctanesulfonic acid). After adjustment for fetal age (appendix p 14), all three bile acids were related with multiple amino acids, citric acid cycle metabolites, and lipids, although lithocholic acid showed an opposite, inverse trend, while positive relationships were observed for tauroursodeoxycholic acid and tauromuricholic acid isomers.

Individual lipids were modestly related to perfluorooctanesulfonic acid and WQS-weighted perfluoroalkyl exposure; however, for perfluorooctanoic acid, perfluorohexanoic acid, and perfluoroundecanoic acid modest negative relationships with several lipids were observed (figure 3C, D). Weighted perfluoroalkyl exposure was inversely related with lipids containing polyunsaturated fatty acids (PUFAs).

All measured perfluoroalkyls were mostly negatively related to various individual polar metabolites (figure 2C, D), with the exception of C4, a known marker of bile acid synthesis rate,[22] which was strongly positively related to weighted perfluoroalkyl exposure and to individual perfluoroalkyls. Here, perfluorooctanesulfonic acid and weighted perfluoroalkyl exposure negatively associate with the lowest number of metabolites, including the amino acids tryptophan, valine, isoleucine or leucine, tyrosine, methionine, and glutamine but also isovaleric acid, itaconic acid, malic acid, and fumaric acid. The pattern of associations of perfluoroundecanoic acid, perfluorohexanoic acid, and perfluorooctanoic acid with polar metabolites was markedly similar (figure 2C).

Metabolic pathway enrichment analysis showed that the main metabolic pathways associated with perfluoroalkyl were related to fatty acid, bile acid, and central carbon metabolism (appendix p 28).

To identify possible transcriptional mechanisms that might explain the observed associations between metabolome and perfluoroalkyl exposure, we examined the associations between hepatic transcriptional changes and perfluoroalkyl exposure, focusing on a subset of genes selected based on the metabolic enrichment pathway analysis (appendix p 9). Joint pathway analysis using both metabolite and RNA sequencing data further verified that perfluoroalkyl exposure was associated with alterations in lipid metabolism, central carbon metabolism, and amino acid metabolism (appendix pp 29–35). All perfluoroalkyls were negatively associated with transcriptional activity of genes encoding for key enzymes of bile acid synthesis such as *CYP27A1*, *CYP3A4*, and *CYP8B1*, with perfluorohexanesulphonic acid and perfluorooctanoic acid showing significant associations ($p<0·05$, false discovery rate$<0·1$; figure 4; appendix p 36). Additionally, *HNF4α* transcript levels showed strong negative association with perfluoroalkyls, and a positive association was identified between perfluoroalkyls and *FXR* transcript. Perfluoroalkyls also showed negative associations with transcript levels of multiple genes encoding lipid metabolism and positive association with fatty acid transporter *Cd36*. Perfluoroalkyls also showed positive associations with transcript levels of multiple genes encoding gluconeogenesis and pentose phosphate pathway.

Discussion

In this study, we have comprehensively characterised the human fetal liver metabolome and transcriptome across different fetal ages and determined fetal hepatic perfluoroalkyl levels for the investigation of the effect of exposure on human fetal liver metabolic pathways. The fetal hepatic perfluoroalkyl concentrations were in agreement with a previous study reporting their levels in the liver samples from stillborn or electively terminated fetuses.[11] That study also reported that the ratio of maternal serum levels versus fetal hepatic levels was in

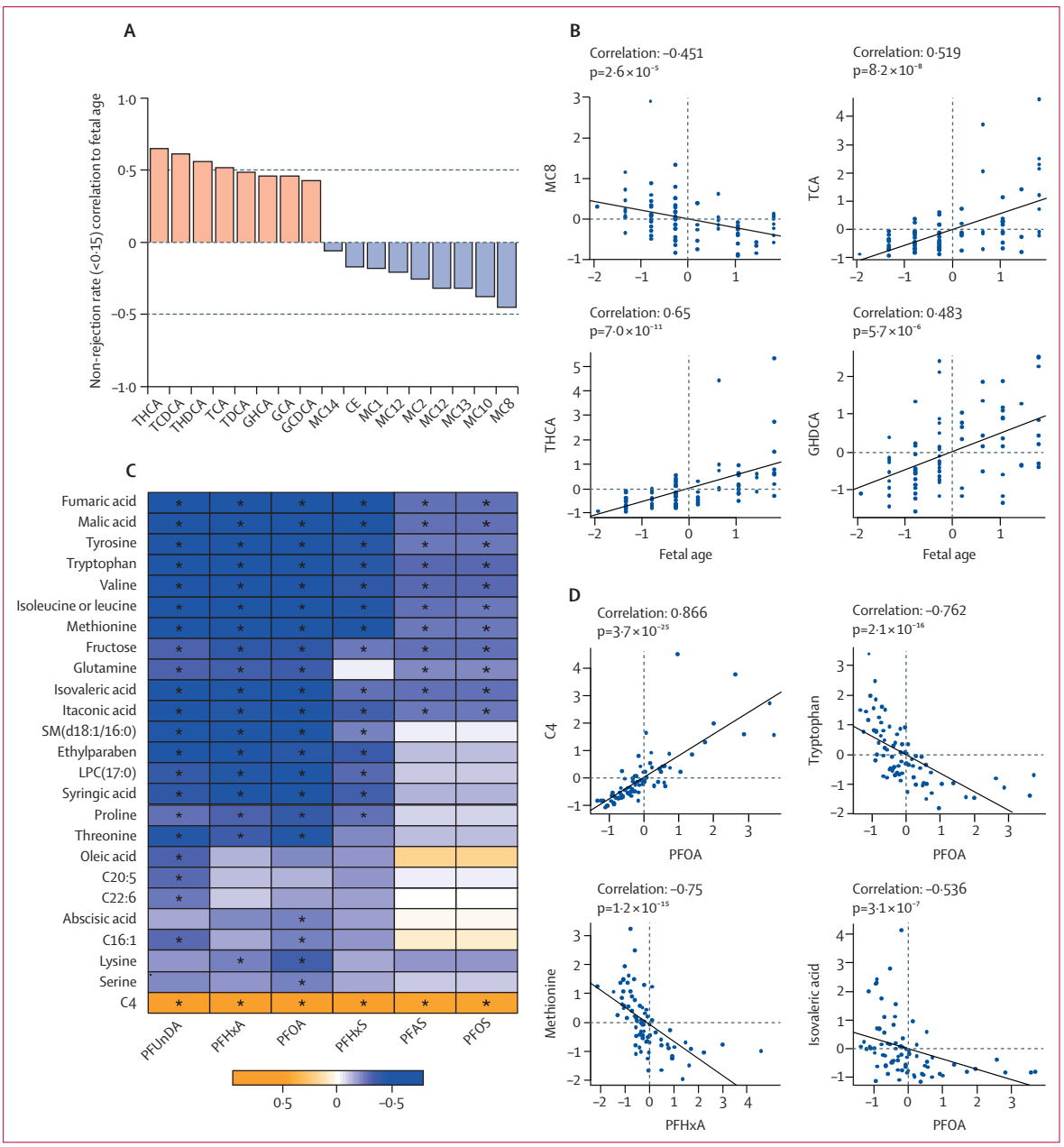
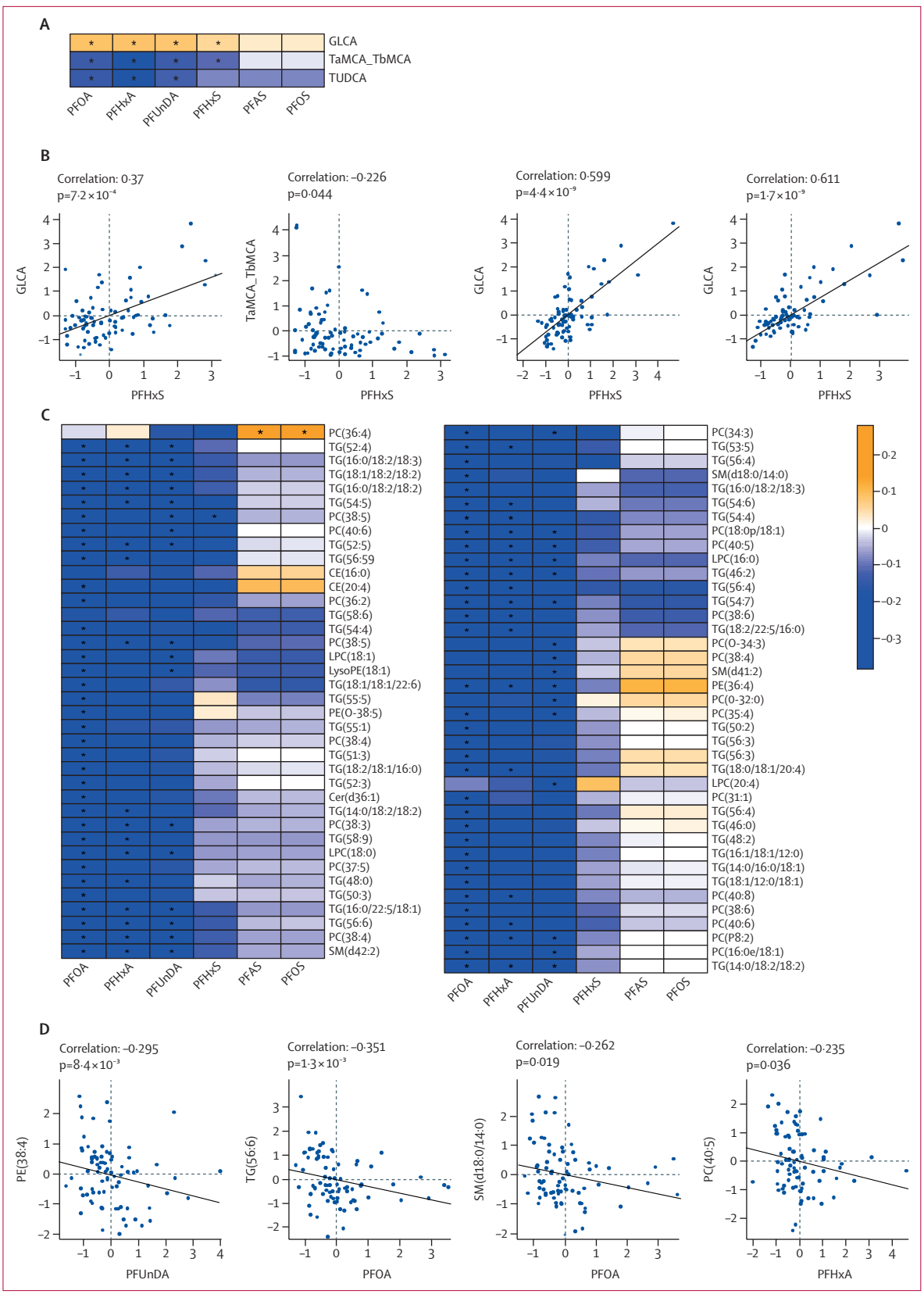


Figure 2: **Fetal age-associated lipid classes and MCs and perfluoroalkyl-associated metabolites**
(A) Bar plot of those study variables (summed lipid classes), MCs, bile acids, and perfluoroalkyls associated with fetal age (gestation weeks), where association is assessed by passing the requirement of a non-rejection rate of less than 0·15. Spearman correlation between the named variable (x axis) and fetal age (y axis) is displayed in orange for positive associations and blue for negative associations. (B) Selected scatter plots depicting the datapoints behind the bar plot in A, whereby individual points represent all samples, a regression line is provided. Values are log2, autoscaled. (C) Heatmap of perfluoroalkyl-associated identified polar metabolites, where association is defined as significant (p<0·05) correlation between fetal age (gestation weeks), BMI of the pregnant woman, and fetal sex-adjusted values for both the polar metabolite and the perfluoroalkyl. Colour bar values represent the Spearman correlation between the variables. Significance of the correlation is denoted by a star in the heatmap cell. (D) Selected scatter plots of the relationships depicted in the heatmap in panel C. C4=7α-hydroxy-4-cholesten-3-one. CE=cholesterol esters. GCA=glycocholic acid. GCDCA=glycochenodeoxycholic acid. GHCA=glycohyocholic acid. GHDCA=glycohyodeoxycholic acid. MC=metabolite cluster. PFAS=perfluoroalkyl substances. PFHxA=perfluorohexanoic acid. PFHxS=perfluorohexanesulfonic acid. PFOA=perfluorooctanoic acid. PFOS=perfluorooctanesulfonic acid. PFUnDA=perfluoroundecanoic acid. TCA=taurocholic acid. TCDCA=taurochenodeoxycholic acid. TDCA=taurodeoxycholic acid. THCA=taurohyocholic acid. THDCA=taurohyodeoxycholic acid. SM(d18:1/16:0)=N-palmitoyl-D-erythro-sphingosylphosphorylcholine. C16:1=palmitoleic acid. LPC(17:0)=1-heptadecanoyl-sn-glycero-3-phosphocholine. C20:5=eicosapentaenoic acid. C22:6=docosahexaenoic acid.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GLCA
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TUDCA
PFOA
PFHxA
PFUnDA
PFHxS
PFAS
PFOS
B
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Correlation: −0·226
p=0·044
Correlation: 0·599
p=4·4×10⁻⁹
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p=1·7×10⁻⁹
GLCA
TaMCA_TbMCA
PFHxS
C
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TG(52:4)
TG(16:0/18:2/18:3)
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TG(56:59
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CE(20:4)
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LPC(18:1)
LysoPE(18:1)
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0·2
0·1
0
−0·1
−0·2
−0·3
D
Correlation: −0·295
p=8·4×10⁻³
Correlation: −0·351
p=1·3×10⁻³
Correlation: −0·262
p=0·019
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p=0·036
PE(38:4)
TG(56:6)
SM(d18:0/14:0)
PC(40:5)
PFUnDA
PFOA
PFHxA

the range of 0·6–11·2.[11] Considering this ratio, the maternal perfluoroalkyl levels in mothers from our study were estimated to be at similar levels as reported in females within the period of our sampling (2004–14).[23,24] Importantly, we identified associations between polar metabolites, lipids, bile acids, perfluoroalkyl, and clinical variables such as fetal age, fetal sex, maternal BMI, and maternal age. The fetal liver metabolome was relatively stable across the gestational age range investigated, with conjugated bile acids and specific fatty acids changing the most. The concentration of bile acids increased with gestational age, suggesting hepatic synthesis of bile acids increases with fetal maturity.

A surprising finding was the strong association between perfluoroalkyl exposure and C4 in the fetal liver. Generally, the classic pathway of bile acid synthesis is initiated by the rate-limiting enzyme CYP7A1, which forms 7α-hydroxycholesterol, subsequently converted to C4. Serum C4 levels are a marker for bile acid synthesis rates (postnatally),[22] although there is little information about its hepatic levels. In our study, we identified a strong positive association between perfluoroalkyls and C4, thus suggesting that this pathway is upregulated with increased chemical exposure. However, in the fetal liver, bile acids are synthesised mainly via an alternative pathway mediated by CYP27A1, which was negatively associated with perfluoropentanoic acid, perfluorohexanesulphonic acid, and perfluorooctanoic acid in our study, indicating suppression of this pathway by perfluoroalkyl. Previously, based on measurements of C4 in plasma of third-trimester fetuses (gestational weeks 22–42), CYP7A1 activity was below the limit of detection in plasma of fetuses younger than 30 weeks of gestation.[25] In our study, *CYP7A1* was also undetected. In contrast, using a sensitive, targeted quantitative method, we report detection of C4 in the fetal livers from as early as at gestational week 12, which might represent maternal C4.

C4 was positively associated with all individual perfluoroalkyls, suggesting that, in contrast to adults, perfluoroalkyl exposures are probably driving increased bile acid turnover in the fetal liver via the classic pathway, plausibly through maternal transport of C4 and in the absence of the suppression of CYP7A1, which is not functional in the fetal liver. In conjunction with this mechanism, glycolithocholic acid, a glycine conjugate of the secondary bile acid lithocholic acid, was positively associated with four of five quantified perfluoroalkyls (perfluorooctanoic acid, perfluorohexanoic acid, perfluoroundecanoic acid, and perfluorohexanesulphonic acid). Glycolithocholic acid and its precursor lithocholic acid are hepatotoxic and have been linked with increased susceptibility to metabolic diseases (eg, metabolic dysfunction-associated steatotic liver disease, diabetes, metabolic syndrome, and metabolic dysfunction-associated steatohepatitis). Fetal liver and adult bile acid metabolism differ substantially, particularly with respect to secondary bile acids, due to the absent fetal intestinal microflora and the assumed fetal absence of enterohepatic circulation of bile acids. Secondary bile acids might, however, be transferred from the mother to fetus, as they have been detected in fetal samples.[18] Secondary bile acids might also be produced in hepatocytes because lithocholic acid has also been detected in human hepatocytes,[26] and data also indicate that during fetal and neonatal life, synthesis of bile acids might occur via an alternate pathway whereby lithocholic acid is an intermediate,[27] thus suggesting that lithocholic acid is not solely produced by gut microbiota.

The observed associations between the three bile acids (glycolithocholic acid, tauroursodeoxycholic acid, and tauromuricholic acid isomers), perfluoroalkyl, and metabolome are probably related to the role of bile acids in regulating lipid and glucose homoeostasis. The opposing direction of metabolic changes could be explained by the differential impact of lithocholic acid, muricholic acid, and ursodeoxycholic acid on *FXR*, the most important nuclear receptor involved in the maintenance of bile acid homoeostasis. FXR is also involved in hepatic lipid, glucose, and amino acid metabolism, with lithocholic acid being an FXR agonist, and both TβMCA and tauroursodeoxycholic acid being FXR antagonists.[28] The RNA-Seq data support these results because opposite associations between *FXR* transcript level and glycine conjugates of lithocholic acid, and between *FXR* transcript level and tauroursodeoxycholic acid and tauromuricholic acid were identified. Competing with antagonistic effects of increased levels of TβMCA and tauroursodeoxycholic acid, perfluoroalkyl showed strong positive association with *FXR* transcript level. This association, linked with negative associations between perfluoroalkyl and genes

Figure 3: **Fetal perfluoroalkyl-associated bile acids and lipids.**
(A) Heatmap of perfluoroalkyl-associated bile acids, whereby association is defined as significant (p<0·05) correlation between the fetal age (gestation weeks), BMI of the pregnant woman, and fetal sex-adjusted values for both the bile acids and the perfluoroalkyl. Colour bar values are the Spearman correlation between the variables. Significance of the correlation is denoted by a star in the heatmap cell. (B) Selected scatter plots of the relationships depicted in the heatmap. Values are log2, autoscaled. (C) Heatmaps of perfluoroalkyl-associated lipids, where association is defined as significant (p<0·05) correlation between the fetal age, BMI of the pregnant woman, and fetal sex-adjusted values for both the lipids and perfluoroalkyls. Colour bar values are the Spearman correlation between the variables. Significance of the correlation is denoted by a star in the heatmap cell. (D) Selected scatter plots of the relationships depicted in the heatmap. CE=cholesterol esters. Cer=ceramide. GLCA=glycolithocholic acid. LPC=lysophospatidylcholine. LysoPE=lysophosphatidylethanolamine. PC=phospatidylcholine. PE=phosphatidylethanolamine. PFAS=perfluoroalkyl substances. PFHxA=perfluorohexanoic acid. PFHxS=perfluorohexanesulfonic acid. PFOA=perfluorooctanoic acid. PFOS=perfluorooctanesulfonic acid. PFUnDA=perfluoroundecanoic acid. SM=sphingomyelin. TaMCA_TbMCA=tauro-α-muricolic acid and tauro-β-muricholic acid. TG=triacylglycerol. TUDCA=tauroursodeoxycholic acid.

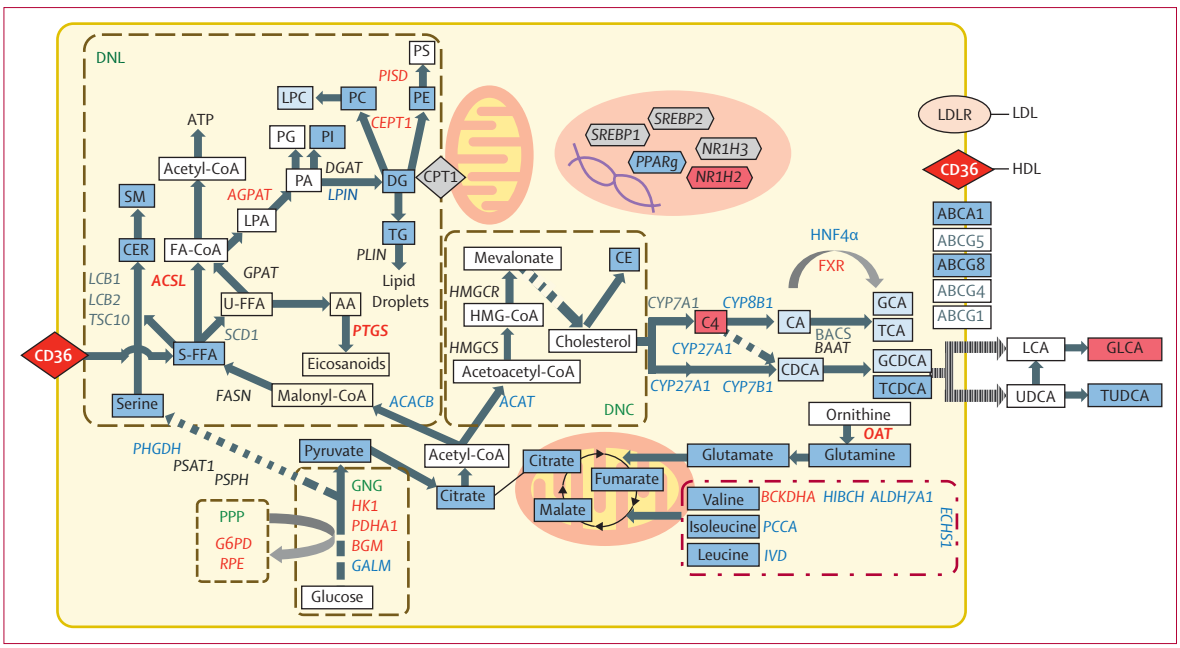

Figure 4: **Metabolic pathways and genes associated with perfluoroalkyl exposure**
Metabolites showing negative associations with perfluoroalkyl are in blue boxes, positively associated metabolites in red boxes, those metabolites showing no significant associations are in grey boxes, and those not measured are in white boxes. Genes showing negative associations are in blue text, positive associations in red text, no significant associations in black text, and genes not detected in grey text. AA=arachidonic acid. C4=7α-hydroxy-4-cholesten-3-one. CA=cholic acid. CDCA=chenodeoxycholic acid. CE=cholesterol ester. CER=ceramide. CPT1=carnitine palmitoyltransferase. DG=diacylglycerol. DNC=de novo cholesterol genesis. DNL=de novo lipogenesis. FA-CoA=fatty acyl CoA. GCA=glycocholic acid. GCDCA=glycochenodeoxycholic acid. GLCA=glycolithocholic acid. GNG=gluconeogenesis. HDL=high-density lipoprotein. LCA=lithocholic acid. LDL=low-density lipoprotein. LDLR=low-density lipoprotein receptor. LPA=lysophosphatidic acid. LPC=lysophosphatidylcholine. PA=phosphatidic acid. PC=phosphatidylcholine. PE=phosphatidylethanolamine. PG=phosphatidylglycerol. PI=phosphatidylinositol. PPP=pentose-phosphate pathway. PS=phosphatidylserine. S-FFA=saturated free fatty acid. SM=sphingomyelin. TCA=taurocholic acid. TCDCA=taurochenodeoxycholic acid. TG=triacylglycerol. TUDCA=tauroursodeoxycholic acid. UDCA=ursodeoxycholic acid. U-FFA=unsaturated free fatty acid.

related to primary bile acid metabolism, is in agreement with the data showing that activation of the FXR pathway induces expression of target genes such as the inhibitory factor *SHP1*, resulting in inhibition of *CYP7A1* and *CYP8B1* transcription.[29] Furthermore, the observed perfluoroalkyl-associated repression of *HNF4A* could be responsible for the repression of these genes, as also reported in human hepatocytes.[30] Metabolic studies also show that ursodeoxycholic acid (UDCA) and its conjugates have hepatoprotective effects by regulating amino acid and fatty acid metabolic pathways.[31] This effect might be linked with the reported perfluoroalkyl-associated changes in amino acid and lipid metabolism in humans.[8] UDCA-treated mice show decreased hepatic levels of total hepatic triglycerides and cholesterol, and upregulated genes involved in fatty acid and cholesterol synthesis.[32] The inverse association between lithocholic acid conjugate and the lipid classes identified in our study agree with studies in animal models that showed that lithocholic acid exposure causes downregulation of circulating lysophosphatidylcholines and sphingomyelins.[33]

The inverse associations between perfluoroalkyl concentrations and PUFA-containing lipids, both in free and esterified forms, might adversely effect fetal development, given the crucial role of PUFAs as the building blocks of cellular membranes, retina, and brain, in structural growth during the third trimester.[34] Because fetuses rely on transfer of PUFAs through the placenta and umbilical cord, decreased PUFA-containing lipids suggests reduced placental PUFA transport. Placental PUFA transfer is dependent mainly on plasma membrane-bound proteins that bind free fatty acids and transport them across the membrane.[34] Some fatty acids cross the placental membrane via passive diffusion; however, these fatty acids are processed and esterified before their release into the fetal circulation.[34] The hypothesis that reduced fetal PUFA-lipids are most probably due to reduced maternal levels is supported by our earlier study showing that in pregnant women, perfluoroalkyl exposure was associated with reduced levels of several free fatty acids and PUFA-containing phospholipids.[17] The inverse association of perfluoroalkyl with multiple lipid classes is in agreement with the previously reported data.[8]

In addition to reduced levels of PUFA-containing lipids, we found other lipid metabolism pathways were associated with in utero exposure to perfluoroalkyl, suggestive of broader changes in fetal hepatic metabolism. Our results are in agreement with previous studies reporting changes in lipid and fatty acid metabolism associated with perfluoroalkyl exposure, both in humans[8]

as well as in animal studies.[35] Furthermore, a 2023 meta-analysis using 11 transcriptomics datasets showed that exposure to perfluoroalkyl molecules induces transcriptional changes across different species, including humans, and different tissues, with dysregulation of lipid metabolism, immune response, and hormone pathways.[36] Perfluoroalkyls are thought to affect acetyl-CoA carboxylase alpha and acetyl-CoA carboxylase beta, altering the flux of acetyl-CoA to malonyl-CoA conversion.[37] This mechanism consequently generates a larger pool of available acetyl-CoA for lipid and cholesterol synthesis, thereby inducing dysfunctional lipid balance and flux. The perfluoroalkyl-associated lipid and fatty acid changes could also be attributed to the effects of perfluoroalkyl on PPAR nuclear receptors, in addition to effects via hepatic FXR. The latter is probably having a larger role, based on the transcriptomics data, with weaker associations between perfluoroalkyl and PPAR-coding genes compared with the perfluoroalkyl-*FXR* associations. PPARs have also an important role regulating metabolism, particularly fatty acid synthesis and degradation,[14] with placental roles in hormone, glucose, and lipid metabolism. The main PPAR ligands are fatty acids, but they can also be activated by xenobiotics structurally similar to lipids, such as perfluoroalkyl.[38] Importantly, direct testing of effects of perfluoroalkyl on murine fetuses pointed to PPAR transactivation in fetal liver and brain, identifying similar pathways to those being perturbed as in our study.[39] A further indication of the developmental effect of perfluoroalkyl exposure on lipid metabolism was the strong positive association between perfluoroalkyl and CD36, corroborated in animal models.[40] CD36 regulates hepatocyte uptake of fatty acids, and might be a major contributor to metabolic dysfunction-associated steatotic liver disease in adults.[41]

The association between perfluoroalkyl and amino acid or urea metabolism in utero could be due to alteration in amino acid metabolism or alteration in maternal transport of the amino acids, or, plausibly, a combination of both. Alteration of amino acid metabolism has been reported in other studies, such as increased indicators of liver stress and increased levels of specific amino acids in children with elevated perfluoroalkyl levels.[4] Perfluoroalkyl exposure has also been associated with decreased levels of amino acids in pregnant women.[17] Reduced levels of amino acids might have major implications for fetal development, because, together with lactate, they constitute the largest carbon sources for the fetal liver. Amino acids are also critical oxidative substrates, and the accretion of amino acids into proteins is an essential component for fetal growth.

There are multiple sources of perfluoroalkyl, making these compounds ubiquitous. Direct exposure to perfluoroalkyl via diet,[42] indoor air, and house dust[43] are the major contributors of perfluoroalkyl exposure in neonatal life.[44] However, strategies to reduce perfluoroalkyl exposure in sensitive life stages, such as fetal life, are challenging. However, avoidance of the use of perfluoroalkyl-containing household products, minimising consumption of seafood and water from perfluoroalkyl-contaminated sources, might be important in reducing maternal exposure to perfluoroalkyls.

Our study has several strengths. First, the unique sample collection of late first and second trimester, normally progressing, human fetal livers. Second, combining this with comprehensive characterisation of the fetal liver metabolome, together with quantitative exposure data, enables the very timely dissection of metabolic consequences associated with perfluoroalkyl exposure. Third, these findings are supported by unique transcriptomics data from most of the fetuses included in the exposure and metabolomic analyses, enabling us to relate findings back to exposure-associated gene and pathway changes. Furthermore, although there have been limited studies of effects of perfluorooctanesulfonic acid or perfluorooctanoic acid compounds on the developing liver and its metabolism, animal studies, such as that by Blake and colleagues,[35] add strong biological and mechanistic plausibility to our findings.

Our study also has some limitations. The sample size was relatively small, although comparatively large for human fetal studies, and collected over a relatively long time period. We measured metabolic profiles and perfluoroalkyls in the liver only, which might not necessarily be fully representative of other organs.[11] However, our study on metabolic profiles and perfluoroalkyls is the largest cohort of mostly second trimester human fetal livers yet reported. Although the procedure for termination and sample collection was standardised, with tissue sampling performed by the same three trained dissectors and the time of tissue collection before freezing was minimised, it is possible that there might have been metabolic changes in the fetal liver due to the procedure. However, as the methodology was standardised and the same in all fetuses, including the use of the same materials in the collection and storage, these factors are not likely to have major impact on the results related to the effect of perfluoroalkyls on the metabolic profiles. Furthermore, our perfluoroalkyl concentrations in the human fetal liver are similar for the six perfluoroalkyls previously reported.[11] We also did not have socioeconomical data or dietary data from the mothers in the study. However, based on previous studies, maternal diet does not have a very strong association with metabolic profiles in neonates;[17,45] although, mixed results on association between socioeconomic factors (such as maternal education, household income, maternal race, nationality, and country of origin) and perfluoroalkyl levels have been reported.[46] Due to the cross-sectional study design, the possibility of reverse causation cannot be fully excluded. For example, pathologies of the liver and placenta may influence bioaccumulation of perfluoroalkyl.

Although the downstream effects of perfluoroalkyl are a subject of active study, including in early life, by us and others,[5,17] the mechanisms of action through which perfluoroalkyls act upon the human fetus in utero are less well known. Taken together, we report the first profiles of the perfluoroalkyl-associated metabolic perturbations caused in the human fetus, and investigate the biological pathways associated by such exposure. The alterations in the human fetal liver metabolome are plausibly explained by both changes in maternal transport of key metabolic substrates, such as PUFAs and amino acids, and partially by the direct impact on fetal liver metabolism. This work thus underscores the need for action by showing probable metabolic effects of perfluoroalkyl in the fetus as early as 11 weeks of gestation and by showing that perfluoroalkyl exposure can potentially lead to perturbations in lipid metabolism and, particularly, in bile acid synthesis.

Contributors

TH, PAF, and MO designed the study. AM, TH, and MO wrote the manuscript. SSa and AS performed the metabolomic analyses, supervised by TH. AM, TH, and MO performed the statistical analyses and accessed and verified the data. PAF designed the fetal human collection and participated in designing, funding, and performing the RNA-Seq study, with PJO'S, DCH, and JPI. SSh and AD performed and wrote the bioinformatics analysis of the RNA-Seq study. TH, PAF, and MO coordinated the study. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication. The authors are responsible for the study design, data collection, data analysis, results interpretation, and manuscript preparation.

Declaration of interests

DCH is a founder, director, and shareholder in Stemnovate and Stimuliver. All other authors declare no competing interests.

Data sharing

Raw RNA-Seq data and processed gene count data are available from Array Express: accession number E-MTAB-6103 (https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-6103). Metabolomics data are available from the Metabolomics Workbench (http://dx.doi.org/10.21228/M8MQ60).

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