

Exposure to perfluoroalkyl substances and risk of hepatocellular carcinoma in a multiethnic cohort

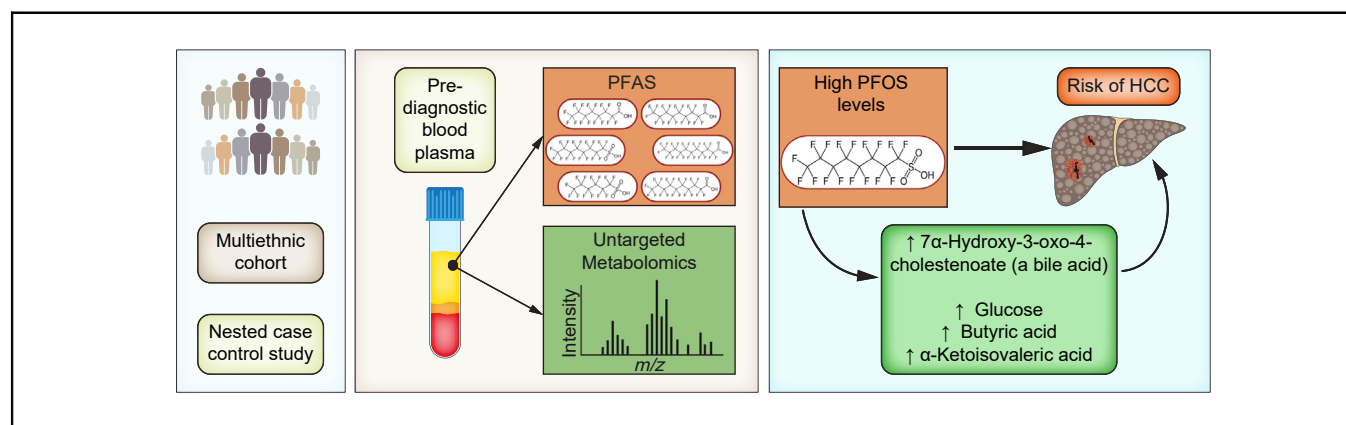
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Graphical abstract



Highlights

- Associations of PFAS and risk of hepatocellular carcinoma were tested in humans.
- PFAS and untargeted metabolomics were assessed in pre-diagnostic samples.
- Exposure to high PFOS levels was linked to increased hepatocellular carcinoma risk.
- The likely mechanisms were via alterations in glucose, amino acid, and bile acid metabolism.

Lay summary

Per- and polyfluoroalkyl substances (PFAS), often referred to as “forever chemicals” because they are difficult to break down and stay in the human body for years, are extremely common and can cause liver damage. In a first of its kind study, we found that exposure to high levels of perfluorooctanesulfonic acid, one of the most common PFAS chemicals, was linked to increased risk of hepatocellular carcinoma in humans. Hepatocellular carcinoma is difficult to treat and is one of the most common forms of liver cancer, and these findings may provide new avenues for helping to prevent this disease.

Exposure to perfluoroalkyl substances and risk of hepatocellular carcinoma in a multiethnic cohort



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Background & Aims: Exposure to poly- and perfluoroalkyl substances (PFAS), a class of persistent organic pollutants, is ubiquitous. Animal studies suggest that PFAS may increase risk of fatty liver and hepatocellular carcinoma (HCC) via impacts on hepatic lipid, amino acid, and glucose metabolism, but human data is lacking. We examined associations between PFAS exposure, altered metabolic pathways, and risk of non-viral HCC.

Methods: In this nested case-control study, pre-diagnostic plasma PFAS and metabolomics were measured in 50 incident HCC cases and 50 individually matched controls from the Multiethnic Cohort (MEC) study. Cases/controls were matched by age, sex, race, and study area. PFAS exposure and risk of HCC were examined using conditional logistic regression. A metabolome-wide association study and pathway enrichment analysis was performed for PFAS exposure and HCC risk, and key metabolites/metabolic pathways were identified using a meet in the middle approach.

Results: High perfluorooctane sulfonic acid (PFOS) levels (90th percentile from NHANES; >55 µg/L) were associated with 4.5-fold increased risk of HCC (odds ratio 4.5, 95% CI 1.2-16.0). Pathway enrichment analysis showed that PFOS exposure was associated with alterations in amino acid and glycan biosynthesis pathways, which were also associated with HCC risk. We identified 4 metabolites linking PFOS exposure with HCC, including glucose, butyric acid (a short-chain fatty acid), α-ketoglutaric acid (a branched-chain α-keto acid), and 7α-hydroxy-3-oxo-4-cholestenolone (a bile acid), each of which was positively associated with PFOS exposure and risk of HCC.

Conclusion: This proof-of-concept analysis shows that exposure to high PFOS levels was associated with increased risk of non-viral HCC, likely via alterations in glucose, amino acid, and bile acid metabolism. Larger studies are needed to confirm these findings.

Lay summary: Per- and polyfluoroalkyl substances (PFAS), often referred to as “forever chemicals” because they are difficult to break down and stay in the human body for years, are extremely common and can cause liver damage. In a first of its kind study, we found that exposure to high levels of perfluorooctanesulfonic acid, one of the most common PFAS chemicals, was linked to increased risk of hepatocellular carcinoma in humans. Hepatocellular carcinoma is difficult to treat and is one of the most common forms of liver cancer, and these findings may provide new avenues for helping to prevent this disease.

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Introduction

Liver cancer was the 6th most common cancer and the 3rd leading cause of cancer death worldwide in 2020.¹ In the United States, liver cancer incidence rates have more than tripled since 1980.² In 2021, liver cancer was the 5th and 7th leading cause of cancer deaths in the US among men and women, respectively.² Hepatocellular carcinoma (HCC) is the most common form of liver cancer, accounting for 85% of cases.¹ With a 5-year survival

rate of less than 20%, HCC is one of the most lethal cancers.³ While global vaccination efforts and effective antiviral treatments have helped to decrease the incidence of HCC related to HBV/HCV infection, the incidence of non-alcoholic fatty liver disease (NAFLD)-related HCC is increasing, and NAFLD is projected to become the predominate cause of HCC in many countries by 2030.⁴ In the USA, the incidence of HCC has decreased since 2011, driven by decreasing HBV and HCV rates.⁵ However, the incidence of HCC in individuals born after 1985 is beginning to match that of baby boomers, which is likely driven by increasing rates of metabolic syndrome, NAFLD, and non-alcoholic steatohepatitis.⁵ Mirroring the increasing global rates of obesity, diabetes, and NAFLD, the incidence of HCC is expected to increase further, and the health impact of this increase is compounded by its dismal prognosis.⁴ Thus, there is an urgent need to identify the risk factors for non-viral-related

Keywords: Chemical exposure; exposome; perfluorinated alkyl substance; hepatocellular carcinoma; metabolome; bile acid; metabolic pathway.

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HCC in order to improve the identification and surveillance of high-risk populations and ultimately reduce the burden of this disease.

Accumulating evidence suggests that poly- and perfluoroalkyl substances (PFAS), a class of pervasive endocrine disruptors, are hepatotoxic.^{6,7} PFAS are persistent and ubiquitous chemicals which have been widely used in industry and consumer products for more than 60 years.^{8–11} In humans, biological half-lives of long-carbon chain PFAS, such as perfluorooctane sulfonate (PFOS), perfluorooctanoate (PFOA), and perfluorohexane sulfonate (PFHxS), range between 3 and 7 years.^{12,13} As such, human biomonitoring studies report widespread exposure with detection rates of over 98% in the blood of US adults.^{14,16,17,43}

PFAS partition preferentially to the liver, where they have hepatotoxic and metabolism disrupting effects.^{6,18,19} Rodents exposed to PFAS, even at low levels, developed liver enlargement, hepatocellular hypertrophy, elevated liver enzymes, and hepatic steatosis.^{20–27} In rainbow trout, PFAS increase the incidence of hepatocellular adenomas and tumors.²⁸ PFAS also alter hepatic lipid, amino acid, and carbohydrate metabolism, which is hypothesized to be a mechanism linking PFAS exposure with hepatotoxic endpoints.²⁹

Despite compelling experimental evidence, investigation of hepatotoxic effects in population studies is limited. Several human studies have examined the cross sectional associations of PFAS exposure with liver enzymes, including of alanine aminotransferase (ALT), a marker of liver function.⁷ For example, in the NHANES study, higher blood PFOS and PFOA concentrations were associated with increased levels of ALT in the general adult population in the USA.^{30,31} In adults, PFAS have also been linked to alterations in cytokeratin 18, a marker for liver apoptosis.^{32,33} Recent evidence also suggests that PFAS are associated with increased risk of NAFLD, diagnosed using liver MRI or liver biopsy, in both children and adults.^{34,35} Using untargeted metabolomics, these studies have also shown that alterations in lipid and amino acid metabolism link PFAS exposure and risk of NAFLD.^{34,35} However, research examining PFAS exposure and HCC in humans is non-existent.

Therefore, we performed the first population-based study to examine the association between PFAS exposure and risk of non-viral HCC in a prospective cohort. We hypothesize that PFAS exposure increases risk of HCC via effects on lipid, amino acid, and carbohydrate metabolism. In a well characterized, ethnically diverse cancer cohort, we examined whether pre-diagnostic levels of PFAS were associated with risk of HCC. We also examined the underlying metabolic pathways linking PFAS exposure with risk of HCC using untargeted metabolomics.

Patients and methods

Study population

This study included 50 incident non-viral HCC cases and 50 controls from the Multiethnic Cohort (MEC) study. Cases and controls were matched by age, sex, race/ethnicity, and study area. MEC is a unique, ethnically diverse prospective cohort of >200,000 African Americans, Latinos, Native Hawaiians, Japanese Americans, and Whites followed since the early 1990s in California and Hawaii.³⁶ Incident cancers including HCC over >20-years of follow-up were identified by the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) program. Comprehensive epidemiologic risk factor data from questionnaires, health condition data from Medicare claims, and

California hospital discharge and mortality information from national mortality databases were also collected. The underlying etiology of HCC was identified using a combination of viral hepatitis testing and Medicare claims and classified into viral (hepatitis B or hepatitis C related), NAFLD, alcohol related, and others as previously described.^{37–39} The breakdown of etiology in HCC cases was 33 (66%) NAFLD, 4 (8%) alcohol related, and 13 (26%) cryptogenic cirrhosis. HCC cases with known viral etiology were excluded from this study. Controls were free from diagnosed liver disease, including metabolic dysfunction-associated fatty liver disease. This study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, and ethics approval for this study was provided by the Institutional Review Boards at the University of Southern California (HS-17-00714) and the University of Hawaii (CHS 9575).

Plasma PFAS

Plasma PFAS concentrations in fasting pre-diagnostic samples were determined using liquid chromatography with high-resolution mass spectrometry (LC-HRMS). Plasma samples were prepared by adding 2.5 μ l of an internal standard solution containing 30 different ¹³C-labeled PFAS compounds to 40 μ l of plasma to obtain final ¹³C-labeled PFAS concentrations of 5 μ g/L. This was followed by adding 80 μ l of ice-cold acetonitrile to precipitate proteins. Treated samples were vortex mixed, equilibrated on ice for 30 mins, and then centrifuged for 15 min at 18,000 \times g, at 4 $^{\circ}$ C. The resulting supernatant was diluted 2:1 with LC-MS grade water and placed in refrigerated autosamplers. Extracts were analyzed using a Vanquish Binary Pump F Ultra Performance Liquid Chromatography (Thermo Fisher Scientific, Rockford, IL, USA), connected to a Q-Exactive HF-X Orbitrap MS system (Thermo Fisher Scientific, Rockford, IL, USA). PFAS analysis was completed using reverse phase chromatography with negative electrospray ionization. Details on method parameters and operation of the LC-HRMS are provided in the following section.

Following analysis of all study samples, accurate mass m/z peaks corresponding to PFAS and matched ¹³C internal standard peaks were extracted and integrated using a mass error threshold of 5 ppm in TraceFinder 5.1. PFAS were quantified by comparing the ratio of the analyte peak and corresponding internal standard to a 6-point calibration curve prepared in charcoal stripped plasma. Each batch of samples included replicate analysis of NIST 1957 and 1958, as well as method and instrumental blanks. Analyte recovery for major PFAS exceed 90%, with detection limits in the low pg/ml range and coefficient of variations less than 15%. Method accuracy was evaluated by comparison to NIST, and through participation in the CTQ AMAP Ring Test for Persistent Organic Pollutants in Human Serum. The limit of detection for plasma PFAS were 0.43 μ g/L for PFOS, 0.01 μ g/L for PFOA, 0.01 μ g/L for PFHxS, 0.01 μ g/L for perfluorononanoate (PFNA), 0.05 μ g/L for perfluoroundecanoic acid (PFUNDA), and 0.01 μ g/L for perfluorodecanoate (PFDA).

Untargeted plasma metabolomics

The plasma metabolome was determined in fasting pre-diagnostic samples using LC-HRMS. To maximize detection of endogenous metabolites, we expanded established methods⁴⁰ to enable analysis using a dual column and dual polarity approach that included analyses with both reverse phase (RP) and hydrophilic interaction chromatography (HILIC). Untargeted analysis was accomplished using a Vanquish Duo liquid

chromatography (LC) system (Thermo Fisher Scientific, Rockford, IL, USA) equipped with dual pumps and columns with independent flow paths interfaced to a Q-Exactive HF-X Orbitrap MS system (Thermo Fisher Scientific, Rockford, IL, USA). The dual LC system was configured to enable parallel analytical separation and flushing using columns with the same stationary phase and mobile phases optimized for positive or negative ionization. To enable analysis using all 4 analytical configurations, all study samples were first analyzed using RP, after which the system was switched to HILIC. Details on sample preparation and LC parameters for each mode are provided below.

Plasma samples were analyzed in batches of 70 study samples with 10 pooled quality assurance/quality control samples. Prior to analysis, samples were thawed at 4 °C and plasma (40 µl for RP, and 30 µl for HILIC) was extracted with ice-cold acetonitrile (80 µl for RP, and 90 µl for HILIC) containing ¹³C-labeled PFAS and internal standards. Treated samples were vortexed for 10 s, equilibrated on ice for 30 min, and then centrifuged for 15 min at 18,000×g, at 4 °C. The supernatant (40 µl for RP, and 30 µl for HILIC) was added to 250 µl LC vials containing water (80 µl for RP) or 1:1 (v/v) water/acetonitrile (90 µl for HILIC analysis) and placed in a refrigerated autosampler. Samples were analyzed with mobile phases optimized for positive or negative ionization. RP analyte separation was accomplished by C₁₈ (TARGA C₁₈ 5 µm 50 × 2.1 mm, Higgins Analytical, Inc, Mountain View, CA, USA) for both positive mode and negative modes. Mobile phases for RP included water and acetonitrile containing 0.1% formic acid for positive mode and 10 mM ammonium acetate and 95/5 (v/v) acetonitrile/water (A) for negative mode. For HILIC, positive ESI analysis was completed using a SeQuant ZIC-HILIC column (3.5 µm, 200 A 4.6 × 50 mm; MilliporeSigma, Burlington, MA, USA), and mobile phase including 0.1% formic acid in water and acetonitrile. For negative mode, a Waters XBridge Amide column (3.5 µm 3.0 × 50 mm, Waters Corporation, Milford, MA, USA) was used with mobile phases consisting of 10 mM ammonium acetate in water adjusted to pH 9.60 with ammonium hydroxide and 95/5 (v/v) acetonitrile. Total runtime for each analysis was 7.5 min. Mass spectral data was collected over the scan range 85–1,275 at 120,000 (FWHM) resolution. Spray voltages were maintained at 3.5 kV and 4.0 kV for positive and negative mode, respectively. Sheath and auxiliary gas temperatures were 300 °C and 250 °C, respectively, while sheath and auxiliary gas flow rates were set to 45 and 25 (arbitrary units). To minimize analyte fragmentation at the source, the RF funnel level was set at 35. In addition to full scan data collection, a subset of samples were selected for data-dependent tandem mass spectrometry (MSMS), which collected MSMS spectra for the top 20 most abundant peaks at MS2 resolution of 15,000 using normalized collision energies of 20, 40, and 60. Metabolite peaks were extracted separately for each chromatography and polarity pair using apLCMS and xMSanalyzer,^{40,41} and metabolite features were uniquely identified based upon detected *m/z*, retention time and peak intensity. Following data extraction, LC-HRMS features were corrected for batch effects using the ComBat algorithm.⁴² Features were excluded from the analysis if they were detected in less than 50% of samples or if the quality control samples after batch correction were greater than 0.3, resulting in 4,360 metabolites for analysis.

Statistical analysis

Differences in participant characteristics between cases and controls were tested using *t* tests for continuous variables and

chi-squared tests for categorical variables. Differences in PFAS levels by education status were tested using linear regression, adjusting for case-control status. Associations between PFAS levels and HCC in the matched case-control set were assessed using conditional logistic regression. Adjusted odds ratios (ORs) and 95% CIs were calculated for each case group compared to the reference group. To examine the potential non-linear association between PFAS exposure and risk of HCC, we performed a preliminary analysis modeling PFAS concentrations using smoothing splines within the conditional logistic regression framework. Based upon visual inspection of these models, we observed a potentially non-linear trend between PFOS and risk of HCC. These associations were driven by high levels of PFOS, so for the main analysis, we categorized all PFAS as high vs. low concentration based on the 90th percentile of exposure in NHANES 1999–2000, the earliest date that PFAS monitoring was performed in NHANES.⁴³ In our data, this corresponded to the 85th percentile for PFOS, and so to maintain consistency, the 85th percentile was used to define high vs. low exposure for all other PFAS.

Metabolome-wide association study

To examine the metabolic pathways linking PFAS exposure and risk of HCC, we performed 2 metabolome-wide association (MWAS) studies. For the first analysis, we performed an MWAS examining the associations between PFAS levels and individual metabolites using linear regression. This analysis was performed independently for each PFAS which was found to be significantly associated with risk of HCC. In these models, metabolites were the dependent variable and PFAS concentrations were the independent variable. In order to account for oversampling due to the matching case-control design, models were adjusted for the matching variables, including case/control status, age at blood draw, sex, race/ethnicity, and study area. For the second analysis, we performed an MWAS examining the associations between individual metabolites and HCC status using conditional logistic regression. Prior to analysis, all metabolites were scaled to have a mean of zero and a standard deviation of one.

Metabolite annotation and pathway enrichment analysis

Based on the results from the MWAS analysis, we performed a pathway enrichment analysis using both the mummichog algorithm⁴⁴ and the gene set enrichment algorithm,⁴⁵ implemented in version 2 of the peaks to paths module from MetaboAnalyst version 5.0.⁴⁶ The enrichment analysis was performed independently using the results from both the PFAS to metabolite MWAS as well as the metabolite to HCC MWAS. For this analysis, all LC-MS features from both the positive and negative ion modes were included. The mass tolerance was set to 5.0 ppm, the *p* value for significant features was set to 0.05, and we included retention time in the analysis to improve metabolite annotation. Pathways which were independently associated with both PFAS exposure and risk of HCC were selected for additional analysis. Metabolites from overlapping significant pathways were identified using a meet in the middle approach.⁴⁷

Sensitivity analysis

In order to examine the impact of different modeling assumptions on our main findings, we performed the following sensitivity analyses. First, in order to examine the effect of modeling PFAS exposure as a categorical variable, we performed a supplemental analysis looking at the association between PFAS

exposure and risk of HCC using conditional logistic regression, and modeling PFAS exposure as a continuous variable scaled to a mean of zero and a standard deviation of one. Second, since conditional logistic regression is prone to bias in small sample sizes,⁴⁸ we reran our main analysis using ordinary logistic regression, and controlling for the matching variables (age, sex, race/ethnicity, and study area). Third, due to uncertainties about whether obesity and diabetes mellitus may be on the causal pathway linking PFAS exposure with risk of HCC, we performed a supplemental analysis by rerunning the main analysis with either baseline BMI or baseline diabetes mellitus status as covariates.

Results

Characteristics of the study population

Participant characteristics of HCC cases and controls can be found in Table 1. Because of the matching design, age at blood collection, sex, race/ethnicity, and study area were not different between cases and controls. Compared to controls, HCC cases were more likely to be overweight or obese ($p = 0.003$) and had higher prevalence of diabetes mellitus ($p = 0.01$). Smoking status and alcohol intake were similar in cases and controls.

Table 1. Characteristics of HCC cases and controls from the MEC.

	HCC cases (n = 50)	Controls (n = 50)	p value
Age at blood collection, mean ± SD	69.7 ± 7.37	69.2 ± 7.42	0.76
Years between blood collection and diagnosis, Median (range)	7.2 (0.9, 16.4)	—	—
Sex			—
Male	62%	62%	
Female	38%	38%	
Race/ethnicity			—
White	18%	18%	
African American	6%	6%	
Japanese American	38%	38%	
Latino	24%	24%	
Native Hawaiian	14%	14%	
Study area			—
California	36%	36%	
Hawaii	64%	64%	
Education			0.87
Some high school	18%	16%	
Graduated high school	20%	24%	
Vocational/some college	32%	36%	
Graduated college/graduate/professional school	30%	24%	
BMI (kg/m ²)			0.003
<25	18%	38%	
25-30	36%	46%	
≥30	46%	16%	
Alcohol intake (g/day)			0.58
0	48%	58%	
<12	30%	26%	
≥12	22%	16%	
Smoking status			0.35
Never	26.0%	38.0%	
Former	62.0%	48.0%	
Current	12.0%	14.0%	
Diabetes mellitus			0.001
No	62%	92%	
Yes	38%	8%	

Cases and controls matched on age, sex, race/ethnicity, and study area. Differences in participant characteristics between cases and controls were tested using *t* tests for continuous variables and chi-squared tests for categorical variables. HCC, hepatocellular carcinoma; MEC, Multiethnic Cohort.

Table 2. Geometric mean or 75th percentile of serum concentrations of PFAS (in µg/L) in HCC cases and controls.

PFAS	Controls (n = 50)	Cases (n = 50)
PFOS, GM (GSD)	29.2 (1.95)	29.2 (2.37)
PFOA, GM (GSD)	4.78 (1.89)	4.21 (2.13)
PFHxS, GM (GSD)	2.07 (2.25)	1.84 (3.11)
PFNA, GM (GSD)	0.827 (1.85)	0.844 (2.05)
PFDA, GM (GSD)	0.278 (2.84)	0.27 (2.97)
PFUnDA, 75th percentile	*	0.89

GM, geometric mean; GSD, geometric standard deviation; HCC, hepatocellular carcinoma; PFAS, perfluoroalkyl substances; PFDA, perfluorodecanoate; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoate; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; PFUnDA, perfluoroundecanoic acid.

* Geometric mean not calculated for PFAS with >40% of samples below limit of detection.

Geometric mean and selected percentiles of serum concentrations of legacy PFAS (in µg/L) in HCC cases and controls can be found in Table 2. PFOS, PFHxS, PFOS, PFDA, PFNA were detected in all participants. PFUnDA was detected in 29% of all participants. Plasma levels of all PFAS were similar to those reported in NHANES survey years 1999–2000 and 2003–2004.⁴³ PFAS ranged from uncorrelated to highly correlated, with Spearman correlation coefficients ranging from –0.03 to 0.87 (Fig. S1). There were no significant differences in PFAS levels by education status, an indicator of socioeconomic status (p values ranging from 0.26–0.99).

Plasma PFAS were associated with risk of HCC

We observed a positive association between pre-diagnostic plasma PFAS concentrations and risk of HCC (Table 3). The strongest association was between PFOS and HCC. Plasma PFOS concentrations >54.9 µg/L were associated with 4.5 times higher odds of HCC (95% CI 1.20–16.00; $p = 0.02$). This threshold corresponded to the 90th percentile of exposure in NHANES 1999–2000.⁴³ PFUnDA levels greater than 1.22 µg/L were associated with 2.2 times higher odds of HCC, although this association did not reach statistical significance (95% CI 0.92–5.50; $p = 0.07$).

The plasma metabolome was linked to PFAS exposure and to risk of HCC

Based on the results of the exposure outcome analysis, we examined the associations between high levels of PFOS and the plasma metabolome using an MWAS. Of the 4,361 metabolomics features included in the MWAS, 433 (9%) were associated with high levels of PFOS exposure at a $p < 0.05$, and 6 metabolites were associated with PFOS exposure at a false discovery rate-corrected $p < 0.2$. Functional pathway analysis of the MWAS results

Table 3. Odds ratios and 95% CIs evaluating pre-diagnostic serum concentrations of PFAS and risk of HCC in the MEC.

PFAS	µg/L	Odds ratio (95% CI)	p value
PFOS	>54.9	4.50 (1.20, 16.00)	0.02
PFHxS	>4.3	1.10 (0.56, 2.30)	0.72
PFOA	>8.6	1.20 (0.52, 2.80)	0.67
PFDA	>0.8	0.80 (0.31, 2.00)	0.64
PFNA	>1.5	1.20 (0.49, 3.20)	0.64
PFUnDA	>1.2	2.20 (0.92, 5.50)	0.07

Effect estimates were calculated using conditional logistic regression to account for the matched case-control study design. HCC, hepatocellular carcinoma; MEC, Multiethnic Cohort; PFAS, perfluoroalkyl substances; PFDA, perfluorodecanoate; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoate; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; PFUnDA, perfluoroundecanoic acid.

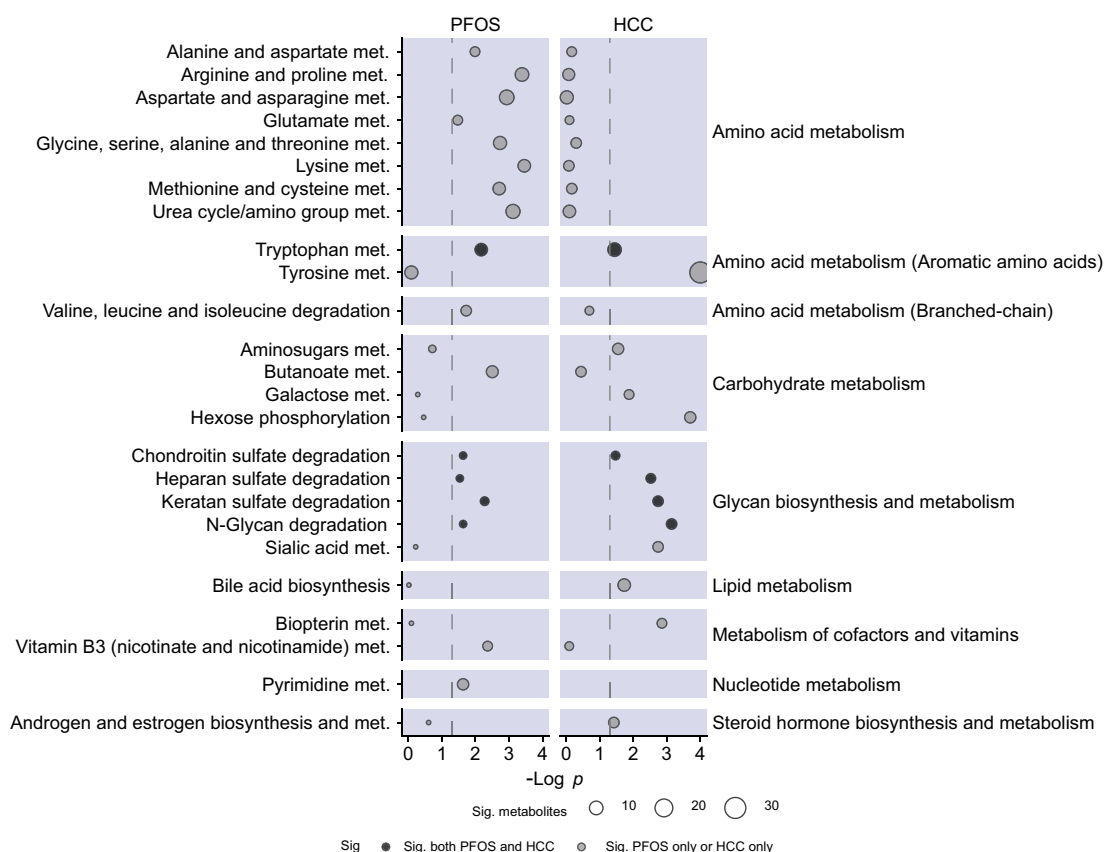


Fig. 1. Metabolic pathways associated with exposure to high levels of PFOS (on left) or HCC (on right) in 50 cases and 50 controls from the MEC cohort. Metabolic pathways are grouped into super pathways as indicated on the right of the plot. Metabolic pathway enrichment was performed using MetaboAnalyst version 5.0. Point size is proportional to the number of significant metabolites associated with each pathway. HCC, hepatocellular carcinoma; MEC, multiethnic cohort; PFOS, perfluorooctane sulfonic acid.

identified significant enrichment of 18 metabolic pathways (Fig. 1). These pathways were primarily related to the metabolism of amino acids, the metabolism of carbohydrates, and glycan biosynthesis and metabolism (Fig. 1).

For HCC, 499 (11%) of LC-MS features were associated with HCC at a $p < 0.05$, and 109 LC-MS features were associated with HCC at a false discovery rate-corrected $p < 0.2$. Functional pathway analysis of the MWAS results identified significant enrichment of 13 metabolic pathways (Fig. 1). These pathways were primarily related to carbohydrate metabolism, glycan biosynthesis and metabolism, and aromatic amino acid metabolism (Fig. 1).

Five metabolic pathways were enriched for both PFOS and HCC. These included tryptophan metabolism, keratin sulfate, heparin sulfate, chondroitin sulfate, and N-glycan degradation. Additionally, 4 metabolites were positively associated with both PFOS exposure and risk of HCC (Fig. 2). These included glucose, butyric acid (a short-chain fatty acid), α -ketoisovaleric acid (a branched-chain α -keto acid), and 7 α -hydroxy-3-oxo-4-cholestenate (a bile acid).

Sensitivity analysis

When modeling PFAS exposure as continuous, we observed a similar positive association between PFOS and risk of HCC, although this association did not reach statistical significance (OR 1.2; 95% CI 0.91–1.60; $p = 0.18$; Table S1). When analyzing our results using ordinary logistic regression and controlling for

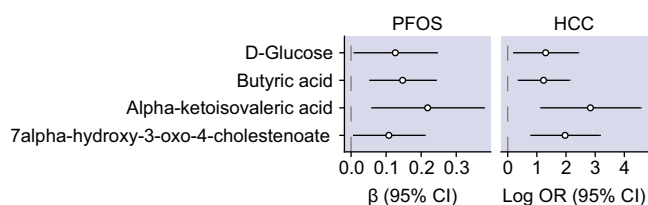


Fig. 2. Effect estimates for metabolites associated with high levels of PFOS and risk of HCC in 50 HCC cases and 50 controls from the MEC. For PFOS exposure, effect estimates were calculated using linear regression adjusting for age, sex, race/ethnicity, and study site, and indicate the mean difference and 95% CI in the \log_2 -transformed metabolite intensity between high ($\geq 85^{\text{th}}$ percentile) vs. low levels of PFOS exposure. Effect estimates for HCC were calculated using conditional logistic regression, and indicate the OR and 95% CI for the risk of HCC per doubling of pre-diagnostic metabolite levels. HCC, hepatocellular carcinoma; MEC, multiethnic cohort; OR, odds ratio; PFOS, perfluorooctane sulfonic acid.

matching variables, we observed a similar positive association between high levels of PFOS and HCC (OR 4.40; 95% CI 1.20–20.00; $p = 0.03$; Table S2). When rerunning the main analysis and accounting for baseline BMI, we observed a similar positive association between PFOS and risk of HCC, although this association did not reach statistical significance (OR 2.90; 95% CI 0.78–10.00; $p = 0.11$; Table S3). When rerunning the main

analysis and accounting for baseline diabetes mellitus, we observed a similar positive association between PFOS and risk of HCC (OR 5.70; 95% CI 1.10–30.00; $p = 0.04$; Table S4).

Discussion

To our knowledge, this is the first prospective study to examine the association between PFAS exposure and risk of HCC. We found that exposure to high levels of PFOS was associated with increased risk of non-viral HCC. Using untargeted metabolomics, we identified several metabolites positively associated with PFOS exposure and risk of HCC. Our findings suggest that PFAS exposure may increase risk of HCC via alterations in glucose metabolism, bile acid metabolism, and metabolism of branched-chain amino acids.

Research examining the associations between PFAS exposure and liver cancer is limited. One existing study has examined the prospective association between PFOA and PFOS concentrations with incident cancer, including liver cancer, in the general Danish population between 1993–2006.⁴⁹ Although this study reported null associations between PFAS levels and risk of liver cancer, a major limitation of this study was that liver cancer was not split by cancer type, and etiology of liver cancer was not available. Between 2004–2006, HCC only accounted for 43% of liver cancer diagnoses in Denmark⁵⁰; it was not until after 2007 that the incidence of HCC dramatically increased, which paralleled increases in obesity, diabetes, and NAFLD.⁵¹ Therefore, non-viral HCC cases were likely a small portion of the total liver cancer cases, which may explain the null findings reported in this study.

Studies examining associations of PFAS exposure with risk of other cancers, such as kidney cancer, in the general population have found similar associations to those reported here. For example, in the only existing nested case-control study examining the prospective association between PFAS levels and risk of renal cell carcinoma, PFOS levels $>50 \mu\text{g/L}$ were associated with a more than 2-fold increased risk of developing renal cell carcinoma (OR 2.51; 95% CI 1.28–4.92), and similar associations were reported for PFOA and PFHxS.⁵² These findings are notable due to the similarity in PFOS concentrations associated with risk of HCC in our study. Other epidemiological studies examining associations of PFAS and cancers have occurred in highly exposed cohorts such as the C8 project,⁵³ which can limit the generalizability of findings to different populations. However, participants in the MEC had PFAS exposure levels similar to the general population. MEC participants were recruited in the early 2000s, and the geometric mean of PFOS in our study (29.2 $\mu\text{g/L}$) is close to the geometric mean for PFOS in the 1999–2000 NHANES survey (30.4 $\mu\text{g/L}$, 95% CI 27.1–33.9).

PFAS exposure has been linked to insulin dysregulation and type 2 diabetes,⁵⁴ which is an emerging risk factor for HCC.^{38,55} In the current study, using untargeted metabolomics we found that PFAS exposure was associated with higher fasting glucose levels, and that higher glucose levels were associated with increased risk of HCC. Increased fasting glucose is a hallmark of type 2 diabetes, suggesting that the association between PFAS exposure and risk of HCC may be partially due to effects of PFAS on glucose and/or insulin dysregulation.

In addition to glucose, we identified 3 additional metabolites linking PFAS exposure and HCC. The compound most strongly

associated with both PFOS exposure and with risk of HCC was α -ketoisovaleric acid, a branched-chain ketoacid and a byproduct in the catabolism of branched-chain amino acids.⁵⁶ Recent evidence in rats suggests that branched-chain ketoacids are associated with alterations in insulin secretion, hepatic lipid metabolism, and hepatic steatosis.⁵⁷ Previous studies have also shown that alterations in branched-chain amino acid metabolism may link early life PFAS exposure with risk of liver injury in children.⁵⁸ The second compound which was associated with PFOS and HCC was 7 α -hydroxy-3-oxo-4-cholestenoate, a bile acid synthesized in the liver. Bile acids are steroid acids with important roles in signaling energy availability and nutrient status.⁵⁹ As such, they play important roles in metabolic diseases including obesity, diabetes, and non-alcoholic steatohepatitis.⁵⁹ Recently, increased levels of primary bile acids have been linked to risk of HCC in humans.⁶⁰ Animal and human studies also suggests that PFAS impact bile acid metabolism, increasing risk of liver injury.^{34,61} The third compound associated with both PFOS and HCC was butyric acid, a short-chain fatty acid which is primarily produced via intestinal fermentation. Short-chain fatty acids play an important role in energy sensing and metabolic regulation in a variety of tissues,⁶² and have been implicated in NAFLD.⁶³ While previous research has identified the importance of branched-chain ketoacids, bile acids, and short-chain fatty acids in the etiology of metabolic disorders and liver disease, our results provide the first human evidence that PFAS-associated alterations in these compounds may increase the risk of HCC.

This study has several strengths. The identification of HCC was based on linkages with SEER-based cancer registries, which are over 95% complete.⁶⁴ We included pre-diagnostic levels of PFAS and metabolomics, which decreases the possibility of reverse causality explaining the observed association between PFAS exposure and HCC. Additionally, the use of fasting plasma samples for determination of the untargeted metabolome removes the potential for confounding due to recent dietary intake, especially for metabolites related to glucose or lipid metabolism. The nested case-control study design allowed us to efficiently examine associations of PFAS exposure with HCC. We were able to individually match cases and controls on age, sex, race/ethnicity, and geographical region, removing potential confounding due to these known risk factors for HCC.³⁷ Despite these strengths, there are some limitations worth noting. First, the sample size of 50 cases and 50 controls is a potential limitation. Because of the limited sample size, we were not able to examine effect modification by known risk factors such as race, sex, BMI, or diabetes status. Second, PFAS exposure can change across time, and we were only able to measure PFAS at a single timepoint. However, the long biological half-life of PFAS, and especially PFOS, means that risk of exposure misclassification is low.

This is the first prospective study to demonstrate that PFAS exposure was associated with risk of HCC. Findings from this proof-of-concept study suggest that PFAS exposure may play an important role in the pathology of HCC. These associations may be driven by alterations in branched-chain keto acids and bile acids. These findings may provide a new insight into the mechanisms of environmental-associated liver disease; however, larger studies are warranted to confirm these findings.

Abbreviations

HCC, hepatocellular carcinoma; HILIC, hydrophilic interaction chromatography; HRMS, high-resolution mass spectrometry; LC, liquid chromatography; MEC, Multiethnic Cohort; MWAS, metabolome-wide association; NAFLD, non-alcoholic fatty liver disease; PFAS, perfluoroalkyl substances; PFDA, perfluorodecanoate; PFHxS, perfluorohexane sulfonate; PFNA, perfluorononanoate; PFOA, perfluorooctanoate; PFOS, perfluorooctane sulfonate; PFUnDA, perfluoroundecanoic acid; RP, reverse phase; SEER, Surveillance, Epidemiology, and End Results.

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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

Concept and design: Goodrich, Walker, McConnell, Conti, Chatzi, Setiawan. Acquisition, analysis, or interpretation of data: Goodrich, Walker, Lin, Wang, Lim, McConnell, Conti, Chatzi, Setiawan. Drafting of the manuscript: Goodrich, Walker, Lim, Conti, Chatzi, Setiawan. Critical revision of the manuscript for important intellectual content: Goodrich, Walker, Lin, Wang, Lim, McConnell, Conti, Chatzi, Setiawan. Administrative, technical, or material support: Walker, Lin, Lim.

Data availability statement

The data shown in this article are available from the corresponding authors upon a reasonable request.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhepr.2022.100550>.

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Author names in bold designate shared co-first authorship

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