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Associations between perfluoroalkyl substances and the severity of non-alcoholic fatty liver disease☆

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) has become the leading cause of chronic liver disease worldwide and the determinants driving its severity remain to be elucidated. Perfluoroalkyl substances (PFAS) are synthetic chemical compounds. They are used in commonplace products and persistent in water, soil and the human body. *In vitro* and animal studies suggest a pathogenic role for PFAS in metabolic diseases such as NAFLD.
Objectives: We aimed to evaluate the association between NAFLD severity and serum PFAS concentrations in humans.
Methods: One hundred biopsy-proven NAFLD patients were included with a well-balanced distribution between the different stages of severity: 25 patients with simple steatosis, 25 with early non-alcoholic steatohepatitis (NASH and F0–F1 fibrosis), 33 with fibrotic NASH (NASH and F2–F3 fibrosis), and 17 with cirrhotic NASH (NASH and F4 fibrosis). Liver histological features were evaluated according to the NASH Clinical Research Network classification. Seventeen PFAS were measured by high-performance liquid chromatography coupled with tandem mass spectrometry on serum samples stored at −80 °C.
Results: The median age was 60 years, 61 % of patients were male, 46 % had diabetes and the median body mass index (BMI) was 32 kg/m^2. Long-chain PFAS were associated with steatosis grade ($p = 0.03$). Among the nine PFAS detected in > 50 % of the patients, Perfluoro-n-heptanoic acid (PFHpA) showed significantly higher concentrations in grade 3 steatosis versus grade 1 ($p = 0.02$). Perfluoro-n-dodecanoic acid (PFDoA) concentrations were higher in patients with significant fibrosis ($p = 0.04$) and PFHpA in patients with advanced fibrosis ($p = 0.02$). The association between PFHpA and steatosis grade remained significant in multivariate analysis adjusted for age, gender, BMI, diabetes presence and dyslipidemia ($p = 0.004$).
Discussion: Our study showed a significant association between PFHpA and liver steatosis in NAFLD. According to data available in the literature, PFHpA could be implicated in liver steatosis through β-oxidation and biosynthesis of fatty acids.

Abbreviations: BMI, body mass index; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PFOA, Perfluoro-n-octanoic acid; PFOS, Perfluoro-1-octanesulfonate; PFAS, perfluoroalkyl substances; PFHxS, perfluorohexanesulfonic acid.

☆ Associations between PFAS and NAFLD severity.

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the manifestation of metabolic syndrome in the liver. It corresponds to an accumulation of fat in that organ, a condition called steatosis. When isolated, steatosis remains benign, but in 25 % of cases, it associates with lobular inflammation and hepatocellular ballooning, defining non-alcoholic steatohepatitis (NASH). This latter is the aggressive form of the disease, promoting liver fibrosis accumulation with a potential for evolution to cirrhosis and hepatocellular carcinoma (Friedman et al. 2018; Sanyal et al. 2021). NAFLD is now the leading cause of chronic liver disease worldwide (Friedman et al. 2018; Younossi et al. 2019), but the pathophysiological mechanisms driving the progression from steatosis to NASH and fibrosis remain largely unknown.

Perfluoroalkyl substances (PFAS) are synthetic chemicals widely used by industry to manufacture many commonplace products such as food packaging, non-stick coating for cookware, clothing and much more. Some PFAS may persist for many years, bio-accumulating in animals and humans with harmful effects on human or environmental health and are classified as "persistent organic pollutants", a list of substances regulated worldwide by the Stockholm Convention. Several studies have shown that PFAS can be detected and measured in serum (Averina et al. 2018; Pérez et al. 2013; Salihovic et al. 2018). For example, seven different PFAS were detected in the blood samples of more than 40 % of 744 adults of the Esteban study, and two of them, Perfluoro-n-octanoic acid (PFOA) and Perfluoro-1-octanesulfonate (PFOS), were present in all subjects (Fillol et al. 2021).

The growing exposure of humans to environmental pollutants is one of the hypotheses forwarded to explain the increase in such chronical diseases as obesity, diabetes and dyslipidemia (Chen et al. 2021; Wan et al. 2014). *In vitro* and animal studies have also suggested the implication of PFAS in the progression of liver steatosis to NASH and liver fibrosis (Curran et al. 2008; Son et al. 2008; Yan et al. 2015; Yu et al. 2016). Following the "multiple-hit hypothesis" which suggests that NAFLD pathogenesis could result from multiple factors (such as genetic predisposition, insulin resistance, nutrition, intestinal microbiota, environment, etc), PFAS could be one of these hits. In an Italian cohort, employees working in a factory producing PFAS presented higher rates of mortality, liver cancer and cirrhosis than did those of a nearby metalworking factory (Girardi and Merler 2019). Several other works have found an association between PFAS and impaired biological markers of liver injury such as alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase or cytokeratin 18 (Bassler et al. 2019; Lin et al. 2010; Nian et al. 2019; Salihovic et al. 2018; Stratakis et al. 2020). Only three studies have been conducted in patients with biopsy-proven NAFLD, enabling the study of associations between PFAS and the severity of liver lesions. The first one conducted in 74 obese children from Atlanta (USA) showed that serum PFOS and perfluoro-1-hexanesulfonate (PFHxS) concentrations were higher in NASH than in simple steatosis (Jin et al. 2020). The second study, including 105 obese adults undergoing bariatric surgery in Sweden, showed that PFOS and PFOA content increased with NASH (Sen et al. 2021). In contrast, the third work conducted in 161 obese Finnish patients undergoing bariatric surgery found a negative association between four PFAS (PFOA, PFHxS, Perfluoro-n-nonanoic acid and Perfluoro-n-decanoic acid) and lobular inflammation in the liver (Rantakokko et al. 2015). These conflicting studies were performed in particular settings such as pediatrics or bariatric surgery. Therefore, no firm conclusion can currently be drawn on associations between PFAS and the severity of liver disease in NAFLD.

Thus, for the present study, we aimed to evaluate associations between NAFLD severity and serum concentrations of PFAS in a well-characterized cohort of biopsy-proven patients recapitulating the different stages of liver disease severity.

2. Patients and methods

2.1. Patients

Angers Cohort – Patients included in the present work came from a cohort of adult patients with biopsy-proven NAFLD who were prospectively recruited in the Angers (France) University Hospital (Boursier et al. 2019). That cohort comprised patients who had liver biopsies for suspected NAFLD after exclusion of concomitant use of steatosis-inducing drugs (such as corticosteroids, tamoxifen, amiodarone or methotrexate). The exclusion criteria were the presence of other chronic liver diseases (autoimmune hepatitis, viral hepatitis, drug induced liver injury, hemochromatosis, cholestatic liver disease, Wilson's disease), a history of excessive alcohol consumption (>20 g/day for women; >30 g/day for men), or a history of liver complications (liver failure, encephalopathy, ascites, variceal bleeding, systemic infection or hepatocellular carcinoma). All patients were recruited within a hepatology clinic setting and no biopsies were performed during bariatric surgery. Blood samples were taken the day of the liver biopsy and stored at −80 °C. For the present work, we selected one hundred patients having liver biopsies ≥ 15 mm in length while aiming to obtain an equitable distribution of the different stages of NAFLD severity. The selected patients were initially included between December 2016 and December 2021. Diabetes was defined as antidiabetic medication or fasting glucose ≥ 126 mg/dl. Dyslipidemia was defined as lipid-lowering treatment or triglycerides ≥ 200 mg/dl or total cholesterol ≥ 200 mg/dl. The study protocol conformed to the ethics guidelines of the current Declaration of Helsinki and obtained approval from the local Ethics Committees. All patients gave written informed consent before inclusion.

2.2. Liver biopsy

Pathological examinations were performed by one senior expert (SM) specialized in hepatology and blinded for patient data. Histological lesions were evaluated according to the NASH Clinical Research Network criteria (Kleiner et al. 2005). The grade of steatosis was defined by the percentage of hepatocytes containing steatosis vesicles and scored as: 0: <5%; 1: 5 %–33 %, 2: 34 %–66 %, and 3: >66 %. Lobular inflammation was defined by the number of inflammatory foci (containing two or more inflammatory cells) and scored into four categories at 200x field: 0: no foci, 1: <2 foci, 2: 2–4 foci and 3: >4 foci. Hepatocellular ballooning was graded as: 0: none, 1: few balloon cells, and 2: many cells/prominent ballooning. NASH was defined as grade ≥ 1 in each of the three components (steatosis, lobular inflammation, and hepatocellular ballooning). Portal inflammation was graded as: 0: none or rare lymphocytes in portal tracts, 1: mild and 2: moderate to severe (Brunt et al. 1999). Liver fibrosis was staged as F0: no fibrosis, F1: perisinusoidal or portal/periportal fibrosis, F2: perisinusoidal and portal/periportal fibrosis, F3: bridging fibrosis, and F4: cirrhosis. "Significant fibrosis" was defined as fibrosis F ≥ 2 and "advanced fibrosis" as F ≥ 3. The four stages of NAFLD severity, as defined by the EASL guidelines (European Association for the Study of the Liver (EASL) et al. 2016), were also considered: NAFL (steatosis without NASH), early NASH (NASH and fibrosis F0–1), fibrotic NASH (NASH and fibrosis F2–3), cirrhotic NASH (NASH and fibrosis F4).

2.3. Perfluoroalkyl substances

Sample preparation – Serum samples (0.5 mL) were first submitted to overnight, room temperature alkaline digestion using potassium hydroxide (3 mL KOH 0.1 M in methanol). The pH was then adjusted to neutral with 100 mL of glacial acetic acid before centrifugation for 10 min at 4000 rpm. Samples were then evaporated to 0.5 mL under a gentle nitrogen stream in a dry bath at 50 °C and reconstituted with 4 mL of 0.1 M formic acid. Samples were loaded onto Oasis HLB Cartridges

preconditioned with 10 mL of MeOH and 10 mL of 0.1 M formic acid. The cartridge was washed with 5 mL of 0.1 M formic acid and 5 mL of MeOH/0.1 M formic acid (50/50, v/v) before elution of the target compounds with 6 mL of MeOH/ammonium hydroxide (99/1, v/v). Extracts were reduced to 1 mL prior to application on Envicarb cartridges preconditioned with 10 mL of MeOH. Target analyses were eluted with 6 mL of MeOH/glacial acetic acid (80/1, v/v). Final extracts were evaporated to dryness and reconstituted in 200 mL of MeOH/water (30/70, v/v).

Instrumental analysis – Seventeen different PFAS were monitored (Table 1). All PFAS were analyzed using ultra-performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). A Hypersil Gold column (100 mm, 2.1 mm i.d., 1.9 μm thickness) was used for separation with 20 mm ammonium-acetate and methanol as the mobile phase. Detection was operated on a triple quadrupole instrument after negative electrospray ionization (Waters Xevo TQS). Data acquisition was operated under the dynamic multiple reactions monitoring mode, with two diagnostic signals monitored per compound (except perfluorobutanoic acid and perfluoropentanoic acid). Quantification was performed using the isotopic dilution method with labelled internal standard compounds.

QA/QC - The applied methodology was based on a fully validated and ISO 17025 accredited method, already used for years in the concerned National Reference Laboratory in particular within the French human biomonitoring program (Fillol et al. 2021). The method also meets the QAQC criteria defined in international ICI/EQUAS assays, such as the one performed in the frame of the Human Biomonitoring for Europe (HBM4EU) initiative (Nübler et al. 2022). The method includes a number of procedural blank analyses for eventual external contamination assessment and management, systematic QAQC sample analyses as positive control, and a quantification using the isotopic dilution method with ^{13}C labelled internal standard compounds.

2.4. Statistical analysis

Continuous variables were expressed as medians with 1st and 3rd interquartile ranges, and compared with the Mann-Whitney test or the Kruskal-Wallis test when appropriate. Categorical variables were expressed as percentages and compared with the Fisher test. The geometric mean, median, 25th and 75th percentiles and minimum & maximum were estimated for each PFAS as done in the other epidemiological studies (Biosurveillance humaine des substances chimiques de l'environnement au Canada 2010; Coakley et al. 2018; Fillol et al. 2021; National Report on Human Exposure to Environmental Chemicals 2022). Every PFAS value below the limit of quantification was replaced by limit of quantification/$\sqrt{2}$, as previously described (Hornung and Reed 1990). PFAS were considered in terms of whole PFAS, PFAS families, and individual PFAS. Whole PFAS corresponded to the sum of all 17 PFAS measured. PFAS families were defined either by the length of the carbon chain (short or long chain) or by the head group (carboxylate, sulfonate or sulfonamide; Table 1). PFAS measuring below the limit of quantification for more than 50 % of the patients were not analyzed individually.

Univariate analysis was used to evaluate the association between PFAS measured in the serum and each liver histological lesion: steatosis, ballooning, lobular inflammation, portal inflammation, fibrosis, NASH, significant fibrosis, advanced fibrosis, and the four stages of NAFLD severity. Then, linear multivariate regressions (backward regression based on the Akaike information criterion for model selection) were used to identify the histological features associated with each PFAS. The candidate variables were steatosis, ballooning, lobular inflammation, portal inflammation and fibrosis, adjusted on sex, age, diabetes, dyslipidemia and body mass index (BMI). A p value ≤ 0.05 was considered statistically significant. Statistical analyses were performed using R version 3.6.0.

Table 1
List of the 17 perfluoroalkyl substances and their families based on chemical head group or chain length.

Name	Abbreviation	Head group	Chain length
Perfluoro-n-butanoic acid	PFBA	carboxylate	Short
Perfluoro-n-pentanoic acid	PFPeA	carboxylate	Short
Perfluoro-n-hexanoic acid	PFHxA	carboxylate	Short
Perfluoro-n-heptanoic acid	PFHpA	carboxylate	Short
Perfluoro-n-octanoic acid	PFOA	carboxylate	Short
Perfluoro-n-nonanoic acid	PFNA	carboxylate	Short
Perfluoro-n-decanoic acid	PFDA	carboxylate	Long
Perfluoro-n-undecanoic acid	PFUnA	carboxylate	Long
Perfluoro-n-dodecanoic acid	PFDoA	carboxylate	Long
Perfluoro-1-butanesulfonate	PFBS	sulfonate	Short
Perfluoro-1-hexanesulfonate	PFHxS	sulfonate	Short
Perfluoro-1-heptanesulfonate	PFHpS	sulfonate	Short
Perfluoro-1-octanesulfonate	PFOS	sulfonate	Short
Perfluoro-1-decanesulfonate	PFDS	sulfonate	Long
Perfluoro-1-octanesulfonamide	PFOSA	sulfonamide	Short
N-Methyl-Perfluoro-1-octanesulfonamide	N-MeFOSAA	sulfonamide	Short
N-Ethyl- Perfluoro-1octanesulfonamide	N-EtFOSAA	sulfonamide	Short

3. Results

3.1. Characteristics of patients

Table 2 shows the characteristics of the 100 patients included in the present study. Median age was 60 years and 61 % of the patients were male. Median BMI was 32 kg/m^2 with obesity present in 66 % of the patients. Nearly half of the subjects had type 2 diabetes mellitus (46 %). The liver biopsies were of good quality with a median length of 30 mm. Steatosis without NASH was observed in 25 patients, early NASH (14 F0 and 11 F1) in 25, fibrotic NASH (16 F2 and 17 F3) in 33, and cirrhotic NASH in 17. A total of 50 patients had significant fibrosis (F $\geq$ 2) and 34 had advanced fibrosis (F $\geq$ 3). Among the 17 PFAS measured (Table 1), five were detected in 100 % of the patients (PFDA, PFNA, PFOA, PFOS and PFHxS). In contrast, eight PFAS (PFHxA, PFBS, N-MeFOSAA, PFBA, PFOSA, PFPeA, N-EtFOSAA, and PFDS) were detected in less than 50 %

Table 2
Patient characteristics.

	All (n = 100)
Age (years)	60 (50–67)
Male sex (%)	61
BMI kg/m^2 (%)	32 (29–37)
BMI $\geq$ 30 kg/m^2 (%)	66
T2DM (%)	46
Biopsy length (mm)	30 (22–37)
Steatosis grade (1 / 2 / 3, %)	43 / 31 / 26
Ballooning grade (0 / 1 / 2, %)	22 / 36 / 42
Lobular inflammation (0 / 1 / 2, %)	12 / 78 / 10
Portal inflammation (0 / 1 / 2, %)	31 / 33 / 37
NASH (%)	75
NAS score	4 (3–5)
Fibrosis stage (0 / 1 / 2 / 3 / 4, %)	19 / 31 / 16 / 17 / 17
AST (IU/L)	40 (31–55)
ALT (IU/L)	55 (39–82)
GGT (IU/L)	67 (39–119)
Alkaline phosphatases (IU/L)	76 (58–102)
Bilirubin (μmol/L)	10 (7–14)
Platelets (G/L)	217 (171–261)
Albumin (g/L)	44 (42–46)
Prothrombin time (%)	98 (90–103)

BMI, body mass index; T2DM, type 2 diabetes mellitus; NASH, non-alcoholic steatohepatitis; NAS Score, NAFLD activity score; AST, aspartate amino-transferase; ALT, alanine amino-transferase; GGT, gamma glutamyl-transferase. Continuous variables are expressed as medians with first and third quartiles. Categorical variables are expressed as percentages.

of the patients (Fig. 1). The mean concentrations of the nine PFAS detected in more than 50 % of the patients were similar, as previously reported in western countries (Table 3) (Canada 2010; Coakley et al. 2018; Fillol et al. 2021; National Report on Human Exposure to Environmental Chemicals 2022). Mean PFAS concentrations varied from 0.04 ng/mL (PFDoA) to 8.19 ng/mL (PFOS). The concentration of any one PFAS varied greatly from one individual to another. For example, the concentration of PFHxS varied from 0.20 to 41.43 ng/mL (Table 3).

3.2. Association between PFAS and liver lesions

Whole PFAS – The sum of the concentration of the 17 PFAS was not significantly associated with any individual liver lesion (steatosis, lobular inflammation, ballooning, portal inflammation, fibrosis; Table 4). Whole PFAS was also not significantly associated with the presence of NASH ($p = 0.71$), significant fibrosis ($p = 0.72$), advanced fibrosis ($p = 0.99$), or the four stages of NAFLD severity ($p = 0.75$).

PFAS families – There were 13 short-chain and 4 long-chain PFAS (Table 1). The latter were statistically associated only with steatosis grade ($p = 0.03$) (Table 4). Patients with grade 1 steatosis had a higher concentration of long-chain PFAS than those with grade 2 steatosis ($p = 0.007$) (Fig. 2**A**). Short-chain PFAS were not associated with any liver lesion. When PFAS were grouped according to their head group (sulfonate, carboxylate and sulfonamide), no family was associated with any liver lesion (Table 4).

Individual PFAS – Only the nine PFAS detected in more than 50 % of patients (Fig. 1) were evaluated individually (PFDA, PFNA, PFOA, PFOS, PFHxS, PFUnA, PFHpS, PFDoA and PFHpA). Significant associations were observed between steatosis grades and PFHpA, PFNA, PFDA, and PFUnA (Fig. 2**A**). The concentrations of some PFAS significantly fluctuated among fibrosis stages but no linear or dose–response association was identified. PFDoA was significantly increased in patients with significant fibrosis and PFHpA in patients with advanced fibrosis (Fig. 2**B**). No associations were found between individual PFAS and portal inflammation, lobular inflammation, ballooning, NASH, or the four stages of NAFLD severity.

3.3. Multivariate analyzes

The association between PFHpA and steatosis grade remained significant in multivariate analysis adjusted on age, sex, BMI, presence of diabetes and dyslipidemia ($p = 0.004$). The concentration of PFHpA increased by 0.04 ng/mL for individuals with grade 3 steatosis compared to those with grade 1 steatosis, i.e. a nearly doubled average concentration of PFHpA between those two grades (adjusted average concentration of PFHpA in grade 1 = 0.06 ng/mL versus grade 3 = 0.11 ng/mL). None of the other associations previously highlighted in univariate analysis remained significant after adjustment in multivariate analysis.

4. Discussion

The subject of PFAS as endocrine or metabolic disruptors and the roles they may play in chronic diseases has been garnering a substantially increasing amount of interest over the past few years. The study we present here showed an association between PFHpA and steatosis. No other significant results were found regarding the other pollutants and liver histological features. The present work has several strengths that merit mention: whereas most other studies have focused on a very small number of different PFAS, mainly PFOS and PFOA, ours aimed to detect 17 different PFAS with very low limits of quantification. Furthermore, our cohort comprised all of the different stages of severity of NAFLD, in contrast to those of other studies wherein advanced fibrosis or cirrhosis were poorly present (Jin et al. 2020; Rantakokko et al. 2015; Sen et al. 2021). Finally, we included patients with the clinical and biological characteristics generally present in the hepatology clinic setting, while other studies have been based on specific populations, such as children or obese patients undergoing bariatric surgery (Jin et al. 2020; Rantakokko et al. 2015; Sen et al. 2021).

Serum PFAS measurement and quantification rates were reliable in our work. Indeed, the odds of detection and measured concentrations were similar to those previously reported in general populations from France, Italy, the United States, Canada and New Zealand (Canada 2010; Coakley et al. 2018; Fillol et al. 2021; Ledda et al. 2018; National Report on Human Exposure to Environmental Chemicals 2022) (Table 3) and in other studies including NAFLD patients (Sen et al. 2021). For example, PFOA, PFHxS and PFNA were detected in 100 % of the patients in our cohort, a result comparable to those reported in Canadian and other French studies (Canada 2010; Fillol et al. 2021). The concentration of PFOA was 1.95 ng/mL in our work, falling within the range of 1.6 to 2.4 ng/mL reported in other studies (Canada 2010; Coakley et al. 2018; Fillol et al. 2021; Ledda et al. 2018; National Report on Human Exposure to Environmental Chemicals 2022).

PFOA, PFOS and PFHxS were the only PFAS measured in all of the studies with liver biopsies in the literature. Ours found no significant association between these three PFAS and the different liver lesions evaluated. The other studies did highlight significant associations, but in a conflicting manner. Rantakokko *et al.* found a negative association

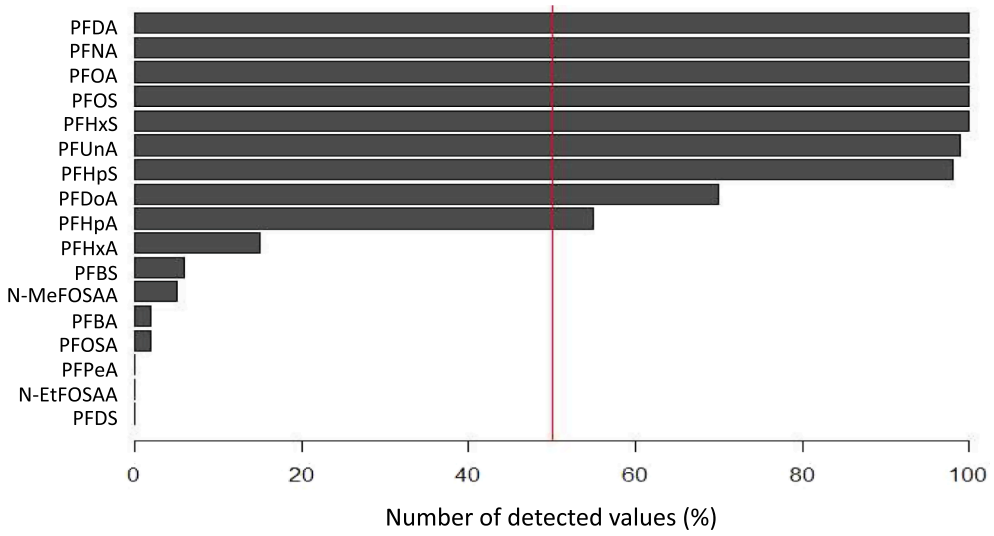

Fig. 1. Rate of patients with PFAS measurements above the limit of quantification.

Table 3
Concentrations of the nine PFAS detected in > 50 % of the patients and comparison to the literature.

	PFHxS	PFHpS	PFHpA	PFOA	PFNA	PFDA	PFUnA	PFDoA	PFOS	TOTAL[#]
Concentration in our cohort										
Geometric mean (ng/mL)	1.58	0.22	0.06	1.95	0.70	0.29	0.15	0.04	8.19	13.18
Standard deviation	1.95	2.18	2.05	1.79	1.81	1.92	1.87	2.29	2.35	
Median (ng/mL)	1.67	0.21	0.07	2.06	0.72	0.28	0.14	0.04	7.61	17.29
1st quartile (ng/mL)	1.16 2.21	0.15 0.34	0.03	1.41	0.49	0.19	0.02	0.02 0.08	5.36 12.13	11.94 24.49
3rd quartile (ng/mL)			0.11	2.75	1.00	0.41	0.22			
Max (ng/mL)	41.43	6.05	0.29	5.69	3.49	2.10	0.77	0.39	229.06	435.33
Min (ng/mL)	0.20	0.01	0.03	0.13	0.13	0.07	0.02	0.01	0.52	1.58
Concentration in other studies from general population (geometric mean)										
France (ng/mL)	1.37	0.18	NC	2.08	0.80	0.34	0.17	NC	NC	NC
Italy (ng/mL)	NC	NC	NC	1.70	NC	NC	NC	NC	NC	NC
New Zealand (ng/mL)	1.00	NC	NC	2.40	0.66	NC	NC	NC	NC	NC
United States (ng/mL)	1.22	NC	NC	1.60	0.59	0.16	NC	NC	NC	NC
Canada (ng/mL)	1.80	NC	NC	2.30	0.82	0.20	0.12	NC	NC	NC

sum of PFHxS, PFHpS, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA and PFOS.
NC: not calculated.

Table 4
Associations between serum PFAS values and liver lesions. Results in the table correspond to p values.

	Whole PFAS	Short-chain PFAS[1]	Long-chain PFAS[2]	Sulfonate PFAS[3]	Carboxylate PFAS[4]	Sulfonamide PFAS[5]
Steatosis	0.48	0.57	**0.03**	0.66	0.15	0.30
Lobular inflammation	0.41	0.42	0.28	0.47	0.59	0.85
Ballooning	0.66	0.68	0.59	0.55	0.63	0.26
Portal inflammation	0.28	0.27	0.56	0.28	0.19	0.17
Fibrosis	0.29	0.33	0.15	0.37	0.21	0.95
NASH	0.71	0.73	0.50	0.70	0.55	0.67
Significant fibrosis	0.72	0.71	0.67	0.46	0.96	0.86
Advanced fibrosis	0.99	0.99	0.82	0.58	0.40	0.58
Four stages of NAFLD severity	0.75	0.76	0.39	0.62	0.78	0.96

[1] short-chain = PFBA + PFPeA + PFHxA + PFHpA + PFOA + PFNA + PFBS + PFHxS + PFHpS + PFOS + PFOSA + N-Me-FOSAA + N-Et-FOSAA.
[2] long-chain = PFDA + PFUnA + PFDoA + PFDS.
[3] sulfonate = PFBS + PFHxS + PFHpS + PFOS + PFDS.
[4] carboxylate = PFBA + PFPeA + PFHxA + PFHpA + PFOA + PFNA + PFDA + PFUnA + PFDoA.
[5] sulfonamide = PFOSA + N-Me-FOSAA + N-Et-FOSAA.

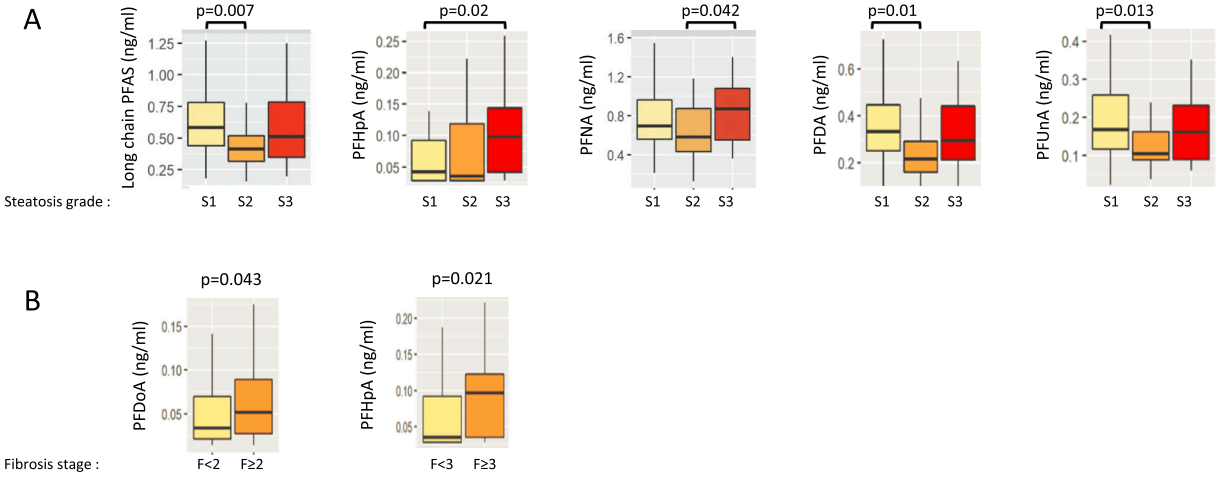

Fig. 2. Significant associations between PFAS and steatosis grades (panel 2A) or liver fibrosis (panel 2B).

between PFOS, PFOA, PFHxS and lobular inflammation (Rantakokko et al. 2015), while Jin *et al.* reported a positive association between PFHxS and lobular inflammation (Jin et al. 2020). Moreover, Sen *et al.* was the only team to find an association between PFHxS and steatosis (Sen et al. 2021). Only one of the four studies (including ours) found an association between fibrosis and PFHxS (Jin et al. 2020). The differences between these studies could be explained by the variability of PFAS concentrations across individuals. Indeed, we and others have found that PFAS concentrations vary widely. For example, PFOS concentrations ranged from 0 to 15.59 ng/mL in the Esteban study (Fillol et al. 2021) and they varied between 0.52 and 229.06 ng/ml in ours. Similarly, in a German study, concentrations of PFOA ranged from 0 to 575 ng/mL (Fromme et al. 2017) while they varied from 0.13 to 5.69 ng/ml in ours. Because the concentrations of these pollutants have also been related to the environment, this inter-individual variability may be related to lifestyles, eating habits, certain jobs and many other still

unidentified factors. For example, in a study on 940 Norwegian adolescents, PFAS concentrations were associated with not only age and BMI, but also the consumption of tobacco and sea products (fat fish, seagull eggs). That study also showed associations between some PFAS and the intake of sugared beverages, junk foods and canned foods (Averina et al. 2018). Another study on 799 Australian firefighters showed that the concentrations of PFHxS and PFOS in the sera of the firefighters were elevated above the 95th percentile of the general population, that elevation surely resulting from exposure to aqueous film forming foam containing PFAS (Evaluation of per- and poly- fluoroalkyl substances (PFASs) in Airservices Australia's Aviation Rescue Fire Fighting Service (ARFFS) Staff – 2018/2019). Unfortunately, we did not have lifestyle or employment information for our cohort but could be for future inclusion.

PFHpA drew our attention in our work because it was associated with advanced fibrosis and steatosis grade, the latter association remaining independent after adjustment in multivariate analysis. We note however that PFHpA was only detected in 55 % of our cohort and at very low concentrations (mean 0.06 ng/mL). Although our PFHpA detection and concentration numbers were similar to those of another study (Salihovic et al. 2018), caution must be exercised when interpreting them. PFHpA is a "new generation" PFAS. Thus, it has not been widely studied and was not mentioned in the three other NAFLD studies with liver biopsies. In a study monitoring 1,002 Swedish individuals over 13 years, changes in the concentration of PFHpA were positively associated with changes in alanine amino-transferase and alkaline phosphatases after adjustment for sex, cholesterol, triglycerides, BMI, fasting glucose levels, statin use and smoking (Salihovic et al. 2018). More generally, PFHpA appears to have a deleterious effect on metabolic diseases. Indeed, it has been associated with increased incidence of gestational diabetes (Rahman et al. 2019; Yu et al. 2021) and diabetes mellitus (Duan et al. 2021), as well as with fasting glucose in pregnant women (Li et al. 2020) and coronary heart diseases (Mattsson et al. 2015). Attema et al.'s study based on transcriptomic results from liver of mice treated with low doses of PFOA during 20 weeks showed an over-expression of gene involved in lipid metabolic process (Attema et al. 2022). In this study, PFOA induced liver steatosis in a peroxisome proliferator-activated receptor alpha (PPARα)–dependent manner. More recently, Yang et al. evaluated a combination of *in silico* toxicological analyses, bioinformatics approaches, animal experiments, and *in vitro* assays to explore the molecular initiating events in PFAS-induced hepatic lipid metabolism disorders (Yang et al. 2023). This study focused on PFOA and PFOS and confirmed an activation of PPARα by these compounds, which in turn activated acyl-coa oxydase 1 (ACOX-1). ACOX-1 promoted peroxisomal lipid metabolism and generated H_2O_2 resulting in oxidative stress that contributed to mitochondrial compromise and lipid accumulation. Reversely, treatment with ACOX-1 inhibitor decreased lipid accumulation in hepatocytes treated with PFOA or PFOS. Other PFAS were also evaluated, and PFHpA was shown to significantly upregulate *Acox-1* expression levels in human hepatocytes. Another study also found an increase in *Acox-1* gene expression following a sub chronic 28-day dermal exposure of PFHpA in a murine model (Weatherly et al. 2023).

In conclusion, we studied 17 different PFAS in 100 well-characterized patients with biopsy-proven NAFLD recruited from within the hepatology clinic setting. We found a significant association between PFHpA and liver steatosis after adjustment on age, sex, BMI, presence of diabetes and dyslipidemia. According to data available in the literature, PFHpA could be involved in liver steatosis through β-oxidation and biosynthesis of fatty-acids.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Clemence CANIVET reports financial support was provided by Association Francaise d'étude du foie.

Data availability

Data will be made available on request.

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