Targeted quantification of perfluoroalkyl substance (PFAS) in human serum from patients evaluated for non-alcoholic fatty liver disease (NAFLD)

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STEEP Sources, Transport, Exposure & Effects of PFASs — University of Rhode Island Superfund Research Program
NIH National Institute of Environmental Health Sciences — Superfund Research Program
THE UNIVERSITY OF RHODE ISLAND

ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is characterized by the accumulation of lipids in the liver, lipotoxicity, and insulin resistance. Obesity, diabetes, genetics, and environmental exposure are considered risk factors for development of NAFLD. Perfluoroalkyl substance (PFAS) are synthetic environmental toxicants, and some are known to be highly persistent and bioaccumulative *in vivo* (i.e., detected in human liver). Perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), have been associated with elevated serum liver injury markers in humans, but few studies have addressed risk of NAFLD. Therefore, this study aimed to compare the serum concentrations of PFOA, PFOS, and emerging PFAS among NAFLD cases (N=23 for children, 17 for adults) and non-NAFLD controls (N=15 for children, 11 for adults). Blood samples were collected at the time of being evaluated for NAFLD between 2014 - 2020 and evaluated for NAFLD biomarkers at Children's Mercy Hospital or the University of Kansas Medical Center. Preliminary findings illustrated that in children, PFOS, PFOA, PFHxS, PFPeA, PFHpA, PFBS, and PFBA accumulated in serum, respectively. For PFOA, PFHxS, and PFOS, the odds ratios (95%) for detection comparing cases to controls were 0.05 (0.002, 1.27), 0.40 (0.03, 4.84), and 0.39 (0.006, 23.59), respectively, adjusted for age, sex, BMI, and race. The averaged log transformed PFOA concentrations in pediatric cases were 0.208 (95% CI: -1.08, 0.66) lower than controls. In the adults, PFHxS, PFOS, PFOA, PFBA, PFBS, PFPeA, and PFDA accumulated in serum, respectively. Differences in log transformed concentration comparing adult cases to controls adjusted for age and sex were: -0.4 (95% CI: -1.10,0.30) for PFOA, 0.05 (95%CI: -0.89, 0.98) for PFHxS, and -0.97 (95% CI: -1.68, -0.25). The findings suggest that children with NAFLD had lower serum PFAS concentrations than children without NAFLD. However, a larger sample size is required to confirm these findings, with sample collection and analysis ongoing.

INTRODUCTION

NAFLD is a spectrum disease – ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) to cirrhosis. According to liverfoundation.org, ~25% of adults suffer from NAFLD, thereby making it the most common chronic liver condition in the USA. Steatosis is the accumulation of triglycerides within the hepatic parenchymal cell cytoplasm, whereas NASH consists of classical steatosis accompanied by inflammation (Reddy et al. 2006). Multiple factors are suggested to contribute to NALFD (**Figure 1**), and PFAS exposure is hypothesized as a potential risk factor for predisposition to NAFLD. PFAS are synthetic environmental toxicants widely used due to their oil-, stain-, and water-repellent properties. The liver is a site of deposition for numerous PFAS in many species, including human (Bassler et al. 2019), rat (Lau et al. 2007), fish (Taniyasu et al. 2003), among others. Exposure to some PFAS has been associated with liver injury which includes apoptosis and altered serum adipocytokines (Bassler et al. 2019) and increased liver function biomarkers (Gallo et al. 2012). According to the EPA, as of 2021 there are ~9,000 PFAS. However, only a handful have been evaluated for human accumulation and biological effects. Therefore, this study aims to understand PFAS accumulation and the link between PFAS exposure and NAFLD biomarkers in humans.

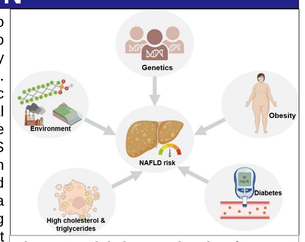


Figure 1. Risk factors for development of NAFLD. NAFLD is characterized by the accumulation of lipids in the liver, lipotoxicity, and insulin resistance. There is no current treatment for NALFD. However, exercise and diet can help reduce and/or reverse disease progression.

HYPOTHESIS

We hypothesized that environmental exposure to PFAS is associated with the risk of developing NAFLD based on the association of some PFAS and elevated serum liver injury markers in humans.

MATERIALS and METHODS

Measurement of serum PFAS concentration via LC-MS/MS. 100 µL of human serum characterized for NAFLD diagnosis (**Figure 2**), was spiked with 12.5 ng/mL MPFAC_MxA PFAS internal standard, digested with 100 µL of 0.1 M formic acid and precipitated with 1 mL of ice-cold acetonitrile. Samples were centrifuged at 10,000 rpm for 5 minutes. The supernatant was transferred to a 15-mL falcon tube and diluted with 9 mL of DI water. Waters™ 3 cc, 50 mg sorbent 60 µm particle size SPE Oasis WAX cartridge (Milford, MA) was used to extract PFAS from serum. The Oasis WAX sorbent is a polymeric weak anion-exchange, reversed-phase, mixed-mode sorbent designed to extract acid chemicals in biological fluids. The WAX cartridge was pre-conditioned with 4 mL of 0.03% ammonium hydroxide in methanol and washed with 4 mL of DI water. 10 mL of sample was loaded and passed through the cartridge at a rate of ~1 drop per second. Next, the cartridge was conditioned with 4 mL of pH 4 acetate buffer (25 mM glacial acetic acid and 25 mM sodium acetate trihydrate), washed with 4 mL of methanol and PFAS were eluted with 4 mL of 0.03% ammonium hydroxide in methanol. Eluent was evaporated down to 0.5-0.2 mL under nitrogen via TurboVap (15 psi at 35 ℃). Samples were diluted 1:4 in 2 mM ammonium acetate buffer in an autosampler vial. Targeted analysis was conducted on a Waters Acquity™ ultra performance LC system coupled to a Quattro Premier XE mass spectrometer with electrospray ionization (ESI) in multiple reaction monitoring (MRM). The mobile phase consisted of 95% DI water: 5% Methanol(A) and 95% methanol: 5% DI water(B). The data was acquired using MassLynx™.

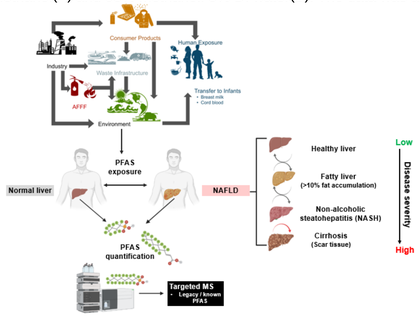


Figure 2. Study scheme for environmental PFAS exposure. Human blood samples of environmentally exposed clinical patients were collected at the time of being evaluated for NAFLD between 2014 - 2020 and evaluated for NAFLD biomarkers (i.e., ALT, AST, BMI) at Children's Mercy Hospital or the University of Kansas Medical Center. NAFLD cases (N=23 for children, 17 for adults) and non-NAFLD controls (N=15 for children, 11 for adults) were assessed for targeted serum quantification of various PFAS (PFOA, PFOS, and emerging PFAS). PFAS exposure scheme was adapted from Sunderland et al. 2019.

RESULTS

Table 1. Per- and polyfluoroalkyl substance (PFAS) characteristics

PFAS Name	Abbreviation	Molecular Weight (g/mol)	Half-life in humans	Manufacturing uses
Perfluorobutanesulfonic acid	PFBS	300.1	665 hours (Olsen et al. 2009)	active ingredient in 3M's new Scotchgard and flame retardant
Perfluorohexanesulfonic acid	PFHxS	400.12	8.5 years (Olsen et al. 2007)	aqueous firefighting foams, textile coating, metal plating and in polishing agents
Perfluorooctanesulfonic acid	PFOS	500.13	5.4 years (Olsen et al. 2007)	fire-fighting foam, carpets, paper and cardboard, textiles and leather and insecticides
Perfluorobutanoid acid	PFBA	214.04	72 - 81 hours (Chang et al. 2008)	stain-resistant fabrics, paper food packaging, carpets and for manufacturing photographic film
Perfluoro-n-pentanoid acid	PFPeA	264.05	Unknown	stain- and grease-proof coatings on food packaging, couches and carpets
Perfluorohexanoic acid	PFHxA	314.05	32 days (Russell et al. 2013)	stain-resistant fabrics, paper food packaging, carpets and for manufacturing photographic film
Perfluoroheptanoic acid	PFHpA	364.06	1.2 years (Zhang et al. 2013)	stain- and grease-proof coatings on food packaging, couches and carpets
Perfluorooctanoic acid	PFOA	414.07	3.8 years (Olsen et al. 2007)	paper and packaging, leather and apparel, carpet, coatings, textiles, rubber and plastics.
Perfluorononanoic acid	PFNA	464.08	4.3 years (Zhang et al. 2013)	food packaging, couches, and carpets
Perfluorodecanoic acid	PFDA	514.08	12 years (Zhang et al. 2013)	food packaging, couches, and carpets

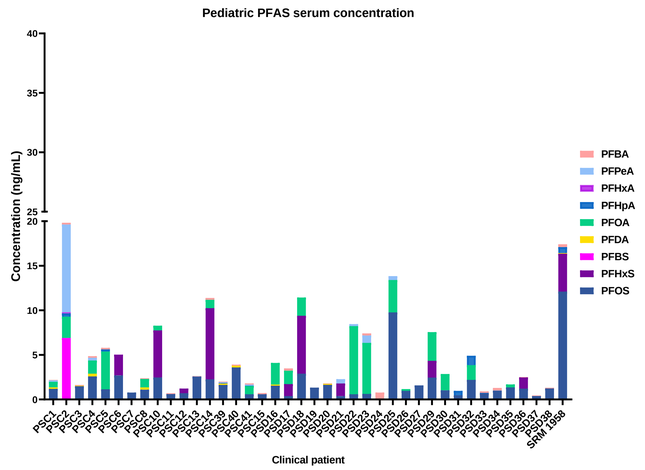
Table 2. Demographic characteristics of pediatric cases and controls

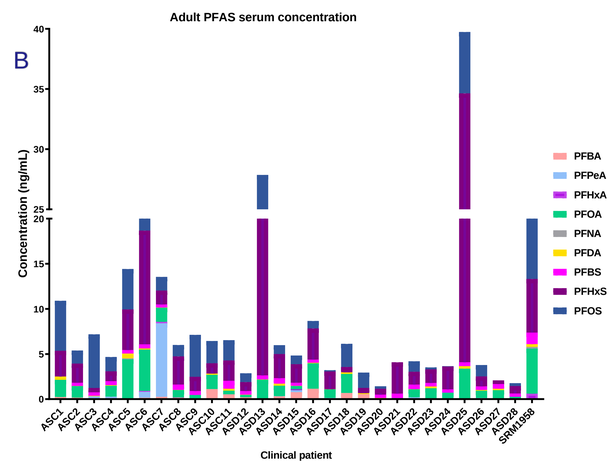
	Case N=23	Control N=16
Age, Mean (SD)	12.43 (3.47)	12.13 (4.16)
Male, N (%)	3 (13.75)	17 (73.91)
Caucasian, N (%)	11 (47.83)	12 (75)
BMI, Mean (SD)	31.70 (8.20)	19.69 (4.16)

Table 3. Demographic characteristics of adult cases and controls

	Case N=17	Control N=11
Age, Mean (SD)	56.41 (15.82)	37.90 (16.95)
Male, N (%)	7 (41.18)	4 (36.36)
Caucasian, N (%)	15 (88.24)	NA
BMI, Mean (SD)	36.66 (8.62)	NA

NA: data not available





Tables 1-3. Study characteristics. Table 1, describes basic characteristics of the targeted perfluoroalkyl acids used for targeted LC-MS/MS quantification. In this study, liquid biopsies were collected from 39 children ages 3-17 at the time of being evaluated for NAFLD between 2014 – 2020 at Children's Mercy Hospital (**Table 2**), and 28 adults ages 24-82 at the University of Kansas Medical Center in 2020 (**Table 3**).

Figure 3. Targeted quantification of PFAS concentration in pediatric and adult serum. A) Summary data (Mean ± SEM) of all measured PFAS in the serum of each pediatric patient – "PFAS fingerprint". B) Summary data (Mean ± SEM) of all measured PFAS in the serum of each adult patient – "PFAS fingerprint".

Table 4. Characteristics of per- and polyfluoroalkyl substances in pediatric serum

Chemical	Overall detection rate, n (%)	Detection rate in controls, n (%)	Serum concentration in controls, mean (ng/mL) ± SEM	Detection rate in cases, n (%)	Serum concentration in cases, mean (ng/mL) ± SEM
PFBA	1 (2.56)	0	<LOQ	1 (4.35)	<LOQ
PFPeA	2 (5.13)	1 (6.25)	2.090 ± 1.938	1 (4.35)	<LOQ
PFHxA	0 (0)	0 (0)	<LOQ	0 (0)	<LOD
PFHpA	1 (2.56)	0 (0)	<LOQ	1 (4.35)	<LOQ
PFOA	17 (43.59)	7 (43.75)	1.594 ± 0.5012	10 (43.48)	2.595 ± 0.6359
PFNA	0 (0)	0 (0)	<LOD	0 (0)	<LOD
PFDA	0 (0)	0 (0)	<LOQ	0 (0)	<LOQ
PFBS	1 (2.56)	1 (6.25)	6.81	0 (0)	<LOD
PFHxS	9 (23.08)	4 (25.00)	4.030 ± 1.645	5 (21.74)	2.482 ± 1.010
PFOS	33 (84.62)	15 (93.75)	1.590 ± 0.2473	18 (54.55)	1.489 ± 0.4050

Table 5. Characteristics of per- and polyfluoroalkyl substances in adult serum

Chemical	Overall detection rate, n (%)	Detection rate in controls, n (%)	Serum concentration in controls, mean (ng/mL) ± SEM	Detection rate in cases, n (%)	Serum concentration in cases, mean (ng/mL) ± SEM
PFBA	6 (21.43)	2 (18.18)	<LOQ	4 (23.53)	~0.5 ± 0.1360
PFPeA	2 (7.14)	2 (18.18)	1.880 ± 1.571	0 (0)	<LOQ
PFHxA	0 (0)	0 (0)	<LOD	0 (0)	<LOD
PFHpA	0 (0)	0 (0)	<LOD	0 (0)	<LOD
PFOA	19 (67.86)	8 (72.73)	1.806 ± 0.4671	11 (64.71)	1.123 ± 0.2485
PFNA	0 (0)	0 (0)	<LOD	0 (0)	<LOD
PFDA	1 (3.57)	1 (9.09)	<LOQ	0 (0)	<LOQ
PFBS	4 (14.29)	2 (18.18)	<LOQ	2 (11.76)	<LOQ
PFHxS	26 (92.86)	10 (90.91)	3.029 ± 1.014	16 (94.12)	4.345 ± 1.944
PFOS	21 (75.00)	10 (58.82)	3.152 ± 0.5275	11 (100.00)	1.519 ± 0.4705

Tables 4 & 5. PFAS characteristics. Table 4, depicts the detection rate of PFAS and mean concentration in pediatric serum with a limit of quantification (LOQ) of 0.5 ng/ mL. **Table 5**, depicts the detection rate of PFAS and mean concentration in adult serum with a limit of quantification (LOQ) of 0.5 ng/ mL. LOD stands for limit of detection.

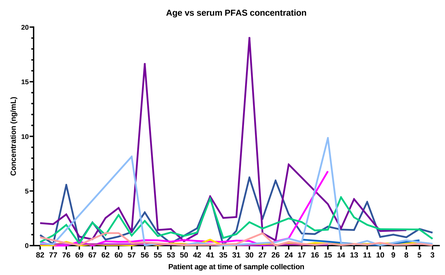


Figure 4. Evaluating PFAS serum accumulation trends based on patient age. PFAS trends associated with decreasing age. Since manufacturing of PFAS began in the late 1940's, the decrease in age helps elucidate legacy PFAS and emerging replacements accumulation trends as a function of time from production until 2014-2020. Data represents the average concentration of each PFAS detected in the serum of at least 2 patients.

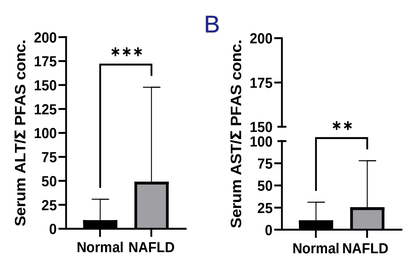


Figure 5. Serum NAFLD biomarker measurement as a ratio of total PFAS concentration in pediatric patients. A) Ratio of clinically measured serum alanine transaminase (ALT) to the sum of total PFAS concentration determined via LC-MS/MS. B) Ratio of clinically measured serum aspartate transferase (AST) to the sum of total PFAS concentration. PFAS concentration represents the geometric mean with geometric standard deviation. Unpaired t test with Welch's correction was performed ($p<0.05$).

CONCLUSION

- For the most prevalent detected PFAS - PFOA, PFHxS, and PFOS, the odds ratios (95%) for detection comparing pediatric cases to controls were 0.05 (0.002, 1.27) p-value 0.07, 0.40 (0.03, 4.84) p-value 0.47, and 0.39 (0.006, 23.59) p-value 0.65, respectively, adjusted for age, sex, BMI, and race.
- The averaged log transformed PFOA concentrations in pediatric cases were 0.208 (95% CI: -1.08, 0.66) lower than controls, adjusting for the same covariates (p-value 0.64). The findings suggest that children with NAFLD had lower serum PFAS concentrations than children without NAFLD. However, a larger sample size is required to confirm these findings, with sample collection and analysis ongoing.
- Differences in log transformed concentration comparing adult cases to controls adjusted for age and sex were: -0.4 (95% CI: -1.10,0.30) for PFOA (p-value 0.27), 0.05 (95%CI: -0.89, 0.98) for PFHxS (p-value 0.92), and -0.97 (95% CI: -1.68, -0.25) for PFOS (p-value 0.008). The findings suggest that adults with NAFLD have a higher PFOA and PFOS concentration than adults without NAFLD.

Acknowledgements

The authors thank Mark J. Strynar for allowing this work to be conducted at the EPA's Campus in Research Triangle Park, Durham, NC and the K.C. Donnelly Externship Award for funding this project. This work was supported by National Institute of Health [grant number P42ES027706].