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Environmental Perfluoroalkyl Acid Exposures Are Associated with Liver Disease characterized by Apoptosis and Altered Serum Adipocytokines

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Abstract

Exposures to perfluoroalkyl substances (PFAS) including perfluoroalkyl acids (PFAAs) are associated with increased liver enzymes in cohort studies including the C8 Health Study. In animal models, PFAAs disrupt hepatic lipid metabolism and induce apoptosis to cause nonalcoholic fatty liver disease (NAFLD). PFAAs are immunotoxic and inhibit pro-inflammatory cytokine release from stimulated leukocytes *in vitro*. This cross-sectional study tests the hypothesis that environmental PFAAs are associated with increased hepatocyte apoptosis and decreased pro-inflammatory cytokines in serum. Biomarkers previously associated with PFAS exposures and/or NAFLD were evaluated as secondary endpoints. Two hundred adult C8 Health Study participants

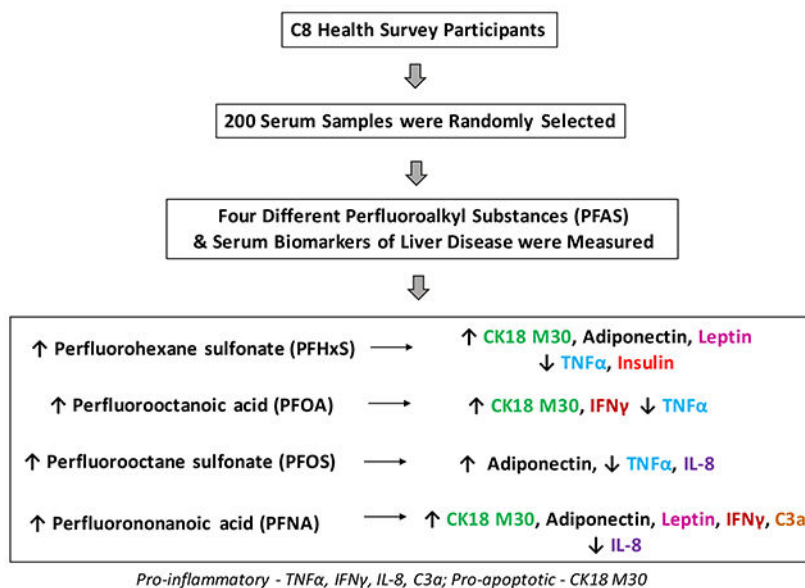
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Conflicts of Interest: Dr. Cave reports financial research support from DiaPharma on an unrelated industry-sponsored clinical trial of the cytokeratin 18 liver disease biomarker. Alan Ducatman was supported to provide health communications for the C8 Health project and has provided support to a plaintiff class seeking medical monitoring following exposure to PFOA. All other authors declare they have no actual or potential competing financial interests.

were included. Measured serum biomarkers included: perfluorohexane sulfonate (PFHxS); perfluorooctanoic acid (PFOA); perfluorooctane sulfonate (PFOS); perfluorononanoic acid (PFNA); cytokeratin 18 M30 (CK18 M30, hepatocyte apoptosis); adipocytokines; insulin; and cleaved complement 3 (C3a). Confounder-adjusted linear regression models determined associations between PFAS and disease biomarkers with cut-offs determined by classification and regression tree analysis. CK18 M30 was positively associated with PFHxS ($\beta=0.889$, $p=0.042$); PFOA ($\beta=2.1$, $p=0.005$); and PFNA ($\beta=0.567$, $p=0.03$). Tumor necrosis factor α (TNF α) was inversely associated with PFHxS ($\beta=-0.799$, $p=0.001$); PFOA ($\beta=-1.242$, $p=0.001$); and PFOS ($\beta=-0.704$, $p<.001$). Interleukin 8 was inversely associated with PFOS and PFNA. PFAAs were also associated with sexually dimorphic adipocytokine and C3a responses. Overall, PFAA exposures were associated with the novel combination of increased biomarkers of hepatocyte apoptosis and decreased serum TNF α . These data support previous findings from cohorts and experimental systems that PFAAs may cause liver injury while downregulated some aspects of the immune response. Further studies of PFAAs in NAFLD are warranted and should evaluate sex differences.

Graphical Abstract



Keywords

Perfluoroalkyl acids (PFAAs); perfluorooctanoic acid (PFOA); non-alcoholic fatty liver disease (NAFLD); cytokeratin 18; tumor necrosis factor alpha (TNF α)

Introduction

Perfluoroalkyl acids (PFAAs) are common manmade compounds within the larger group of per- and polyfluoroalkyl substances (PFAS). They have multiple useful consumer applications including lubrication as well as resistance to grease, stains, and water. Industrial applications include hydraulics, electronics, enhanced extraction of oil and natural gas, and

firefighting. Recently, widespread groundwater contamination has been found at airports and especially military airbases where PFAAs were used for multiple purposes including firefighting foams to extinguish jet fuel fires (Milley et al., 2018; Sunderland et al., 2018). Unfortunately, PFAAs persist in the environment and bioaccumulate in humans. Human exposures occur primarily through ingestion of contaminated drinking water and food. PFAAs have been detected in the serum of almost all people tested in the developed world (Domingo and Nadal, 2017). Longer chain PFAAs have extremely long half-lives in humans (Worley et al., 2017) and accumulate in the liver and other organs (Perez et al., 2013).

PFAS are hepatotoxic, immunotoxic, and endocrine and metabolism disrupting chemicals (DeWitt et al., 2012; Heindel et al., 2017). Environmental cohort studies from North America, Europe, and Asia have consistently reported positive associations between PFAS exposures and liver enzymes (Darrow et al., 2016; Gallo et al., 2012; Gleason et al., 2015; Lin et al., 2010; Salihovic et al., 2018; Yamaguchi et al., 2013). The largest liver enzyme study (n=47,092) found positive associations between alanine aminotransferase (ALT) levels and exposures to perfluorooctanoic acid (PFOA, or C8) and perfluorooctane sulfonic acid (PFOS) (Gallo et al., 2012). The association between PFOA and ALT was subsequently confirmed in 30,723 C8 participants through different techniques; although no associations were found between exposure and self-reported (and subsequently medically validated) liver disease history (Darrow et al., 2016). However, because liver disease may be asymptomatic and subclinical early in its course, the present study applies mechanistic serologic biomarkers to further investigate liver toxicity in the C8 cohort.

Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disease, affecting approximately 25% of the global population (Younossi et al., 2015). NAFLD occurs as a consequence of altered lipid metabolism, resulting in excess lipid accumulation (steatosis) within hepatocytes. PFAA exposures disrupt normal hepatic lipid metabolism to cause steatosis in animal models (Bijland et al., 2011; Das et al., 2017; Kim et al., 2011; Tan et al., 2013; Wan et al., 2012; Zhang et al., 2016). Mechanisms implicated in PFAA-induced steatosis include the upregulation of hepatic *de novo* lipogenesis and lipid uptake from blood (Das et al., 2017) as well as the downregulation of VLDL production/export, possibly due to a reduction of bioavailable choline (Bijland et al., 2011; Zhang et al., 2016).

Hyperlipidemia is the most common comorbid condition associated with human NAFLD (Younossi et al., 2015). PFAA exposures have reproducibly been positively associated with hyperlipidemia in adult and pediatric cohort studies including the C8 Health Study (Frisbee et al., 2010; Geiger et al., 2014; Nelson et al., 2010; Steenland et al., 2009). In contrast to the epidemiological data, PFAA exposures decreased blood lipids in rodents fed a standard diet. However, mice fed a hypercaloric 'Western' diet developed hypercholesterolemia with PFAS treatment (Rebholz et al., 2016). Possible receptor-based modes of action for PFAS-induced alterations in liver and blood lipids include: inhibition of hepatocyte nuclear factor 4 α (HNF4 α); and activation of the xenobiotic receptors, pregnane X receptor (PXR) and constitutive androstane receptor (CAR); the lipid receptors, liver X receptor (LXR) and peroxisome proliferator activated receptors α and γ (PPAR α and PPAR γ); the bile acid receptor, farnesoid x receptor (FXR); and the sex hormone receptor, estrogen receptor α (ER α) (Bjork et al., 2011; Buhrke et al., 2015; Rosen et al., 2017). Importantly, all of these

nuclear receptors have been implicated in NAFLD (Cave et al., 2016). ER α could potentially confer sex differences in PFAS-dysregulated hepatic lipid metabolism and NAFLD; and sex differences have been reported in rodent models (Kim et al., 2011; Rebholz et al., 2016).

Experimental systems demonstrate that PFAS exposures induced hepatocyte caspase 3-mediated apoptosis; increased oxidative stress while reducing NRF2-regulated hepatic antioxidant defenses; and elicited cytoskeletal remodeling (Kim et al., 2011; Wan et al., 2016; Zhang et al., 2016). However, PFAS are potent immunotoxic chemicals which suppress innate immune function. In experimental systems, PFAS reduced LPS-stimulated tumor necrosis factor α (TNF α) production from leukocytes *ex vivo* in whole blood from human volunteers and also *in vitro*, through a partially PPAR α -dependent mechanism (DeWitt et al., 2012). This anti-inflammatory action is consistent with the significantly reduced lobular inflammation associated with PFAS exposures noted on liver biopsy in bariatric surgery patients with NAFLD (Rantakokko et al., 2015). Based on this literature, we postulate that PFAA exposures can cause human NAFLD - associated with hepatocyte apoptosis and paradoxically without increased and possibly with reduced, liver inflammation. This cross-sectional study measures mechanistic serological biomarkers in adult C8 Health Study participants, to test the hypothesis that PFAS exposures are associated with increased hepatocyte apoptosis and decreased pro-inflammatory cytokines. Secondary endpoints included a panel of other serum biomarkers previously implicated in NAFLD and also evaluation of potential sex differences. The significance of this study is that it could help to explain how environmental chemical exposures impact the genesis and progression of liver diseases including NAFLD.

Methods

Serum Specimens and Study Population

The C8 Health Study is a very large population enrollment study from six water districts that were contaminated with perfluorooctanoic acid (PFOA) in the mid-Ohio Valley of Ohio and West Virginia. Recruitment and data collection for the entire C8 Health Study have been described previously (Frisbee et al., 2009). Two hundred serum specimens were randomly selected from the banked specimens of the adult participant population age 40-70 (West Virginia University IRB protocol 190943735), with deliberate oversampling for African American participants and for BMI ≥ 25 in order to obtain a range of BMI. Individuals who reported that they had hepatitis of any origin (viral or otherwise) and from those reporting consumption of >3 alcoholic drinks/day were excluded from the sampling. Serum specimens were stored since 2006 at -80°C and some had undergone freeze-thaw cycle before this investigation was performed in 2017. The sample size (N number) may differ slightly for some measurements due to missing values from the original data set. Missing values are assumed to be missing at random.

Serum Biomarkers of PFAA Exposure

Exposure biomarkers included serum PFOA, PFOS, perfluorohexane sulfonic acid (PFHxS), and perfluorononanoic acid (PFNA). Other PFAS in the data set featured many samples

below the limit of detection and were unsuited for the purposes of this study. The analytical method for PFAS measurement was derived from Flaherty et al. (Flaherty et al., 2005) and has been previously reported (Frisbee et al., 2009; Steenland et al., 2010). Briefly, protein precipitation extraction was followed by reverse-phase high performance liquid chromatography-tandem mass spectrometry in selection mode for the individual PFAS species, compared to a ^{13}C surrogate for quality assurance. Estimates of precision were in the range of $\pm 10\%$.

Serum Disease Biomarkers

The selected serum disease biomarkers were similar to those measured in recent environmental or occupational liver disease cohort studies (Cave et al., 2011; Cave et al., 2010; Clair et al., 2018; Hsieh et al., 2017). Primary serologic outcome biomarkers include the caspase-cleaved cytokeratin 18 fragment (CK18 M30) and pro-inflammatory cytokines. CK18 is a cytoskeletal protein found in epithelial cells including hepatocytes. Dying hepatocytes release CK18 which can then be measured in serum. Levels of the whole protein, CK18 M65, reflect cell death from all causes, while the caspase 3-cleaved fragment (CK18 M30) reflects apoptotic cell death. CK18 M30 is a promising serologic NAFLD biomarker (Feldstein et al., 2009; He et al., 2017). CK18 M30 was positively associated with traffic-related air pollution in overweight and obese children with fatty liver (Hsieh et al., 2017). In contrast, the toxicant associated fatty liver disease (TAFLD) previously associated with VOC and PCB exposures was positively associated with CK18 M65, but not CK18 M30 (Cave et al., 2011; Cave et al., 2010; Clair et al., 2018). This difference is likely related to the predominant hepatocellular death mechanism: necrosis versus apoptosis. Because ALT was previously associated with PFAS exposures in the C8 Health Study (Darrow et al., 2016; Gallo et al., 2012), and animal exposure models demonstrated hepatic steatosis with cytoskeletal remodeling and caspase 3-mediated apoptosis (Kim et al., 2011; Wan et al., 2016); CK 18 M30 was selected as a primary outcome variable for this study. Serum CK18 M30 and M65 were measured by ELISA (Diapharma, West Chester, OH, kits #10011 and 10020) according to manufacturer's instructions using an EPOCH II plate reader running Gen 5 software (Biotek Instruments Inc., Winooski, VT). Measurement of the serum pro-inflammatory cytokines, TNF α , interleukin 6 (IL-6), interleukin 8 (IL-8), and interferon γ (IFN γ), was performed using the Multispot Assay System for Human ProInflammatory Panel II (4-plex kit) per the accompanying manual. The data were acquired and calculated employing the Sector Imager 2400 running Discovery Workbench Software Version 4 (Mesoscale Discovery LLC, Rockville, MD).

Measurement of secondary outcome protein hormones adiponectin, insulin, and leptin was performed using the MSD single spot assay system specific for each analyte. Measurement of cleaved complement 3 (C3a) and plasminogen activator inhibitor-1 (PAI-1) was performed using Human c3a Platinum Elisa Kit and Human PAI-1 ELISA kit (Affymetrix, ThermoFisher, Waltham, MA) according to kit manuals using the EPOCH II plate reader.

Statistical Analysis

SAS 9.4 and R software (version 3.4.4 R Foundation, Vienna, Austria) were used to examine the association between biomarker expressions and PFAS serum concentrations, adjusting

for the potential confounders commonly considered in PFAS studies regarding hepatic effects such as age, alcohol consumption, BMI, sex, and the estimated glomerular filtration rate (eGFR, using the modification of diet in renal disease standard equation) (Levey et al., 2006). Alcohol consumption was categorized as zero or 1-3 drinks per day. Potential subjects with greater alcohol consumption were excluded. Exposure and disease biomarkers were natural log transformed to achieve approximate normality and variance stabilization during the data processing procedure. Exploratory analyses were used to summarize the data, including mean with standard deviation, summary tables and bar-plots. Additionally, a heat map was used to explore the biomarker data simultaneously, and to illustrate which the biomarkers were clustered using hierarchical algorithm with Euclidean distance.

Univariate analysis began with a preliminary test to establish a statistical cutoff level for each biomarker. Using the CART method to express the statistical cut point, a regression tree is built through a process known as binary recursive partitioning, which is an iterative process that splits the data into partitions or branches, and then continues splitting each partition into smaller groups as the method moves up each branch. As a result, the different subgroups are identified to predict the response variable. The cut point for each biomarker is individually assessed using the CART method. From these specified levels, the observed biomarker data are then dichotomized into either high, or low categories, depending on the cut point designated for each biomarker.

The goal of this method is to create a model that predicts biomarker values based on serum PFAS concentrations. The algorithm computes where to prune a tree based on the deviance and constructs a classification tree with the important conditions based on the biomarkers (high *versus* low). Decision tree analysis is increasingly used to support development of algorithms using biomarker screening candidates (Chen et al., 2011; Marshall, 2001). In order to identify a unique cutoff, a summed PFAS mixture concentration was computed based on the normalized z-scores of each PFAS variable; and log-transformed biomarker data were used. Using a t-test, or when necessary based on deviance from statistical assumptions, the Wilcoxon ranked Sum test are implemented to compare the dichotomized levels of each biomarker.

In the multivariate analysis, a linear regression model adjusting for potential confounding variables was used to assess PFAS concentrations by the categorized disease biomarkers. Cognizant of published sex differences in health outcomes associated with PFAS exposures, an interaction term of sex and biomarkers was included in the linear regression model. The results of the multivariate regression model are similar to that of a factorial ANOVA design, with comparable results. Statistical significance was set at both the 0.05 and 0.1 levels. The higher threshold *p* value ($p=0.1$) was included to reduce Type II errors, because this analysis could be considered a pilot study investigating only a small portion of the overall cohort. In all cases, the actual *p* value is provided.

Results

Demographic data are provided in Tables 1A–B. Mean participant age was 55.3 ± 8.4 , 54% were women, and 61% were currently non-drinkers. The oversampling procedures a

distribution of BMI categories (normal weight 40.5%, overweight 27.5%, and obese 32%) as well as a significant number of African Americans (19% vs. 3% in the overall C8 Health Study). Mean serum levels of PFAS (ng/mL) were: PFOA= 94.6±183.6 (SD); PFOS = 26.9±16.7; PFHxS = 4.2±3.9; and PFNA = 1.6±0.7. Mean serum liver enzymes were: alanine aminotransferase (ALT) = 24.0±12.3 (U/L, SD) and aspartate aminotransferase (AST) = 22.8±7.9 (U/L). Serum exposure and aminotransferase biomarkers were similar between sexes (Table 1A).

A heat map of normalized disease biomarkers (Figure 1) demonstrates a visual picture of the correlations between high (red) and low (green) variables. The closest relationship was between the hepatocyte total cell death biomarker (CK18 M65) and the hepatocyte apoptosis biomarker (CK18 M30) as anticipated. Weaker correlations were observed between TNF α and IL-8; IFN γ and IL-6; as well as insulin and leptin. Disease biomarker data were grouped into 'low' or 'high' categories based on cutoffs determined by CART analysis. The cutoff values are provided in the Supplementary Table. The univariate analysis (Supplementary Table) examined the relationships between mean PFAS levels in the 'low' vs. 'high' category for each disease biomarker. Means for univariate and multivariate analyses are compared using a t-test, and when necessary, the Wilcoxon Ranked Sum test is used when t-test assumptions, namely sample size requirements, falter. The Supplementary Figure depicts this information graphically. The multivariate models (Table 2) determined interaction coefficients (Interaction Model) investigating sexually dimorphic responses as well as adjusted beta coefficients (Final Model). Figure 2 provides the same information visually for each of the four evaluated PFAS. The univariate and multivariate results were found to be similar and this relationship is illustrated in Table 3. This table summarizes the results from univariate model between PFAS and biomarkers alone (univariate model) versus multivariable model with additional confounding variables/interaction term.

The primary disease biomarkers were CK18 and pro-inflammatory cytokines. In experimental systems, PFAS exposures were associated with caspase-3 dependent hepatocyte apoptosis such as CK18 (Kim et al., 2011). In the univariate analysis, participants with elevated caspase 3-cleaved CK18 (M30) had significantly higher mean levels of PFHxS, PFOA, PFOS, and PFNA (Supplementary Table). Likewise, those with elevated CK18 M65 had significantly higher mean levels of PFOA, PFOS, PFNA. In the multivariate model, M30 was positively associated with PFHxS (SE) ($\beta=0.889\pm0.434$, $p=0.042$); PFOA ($\beta=2.1\pm0.739$, $p=0.005$); and PFNA ($\beta=0.567\pm0.262$, $p=0.03$) with a trend for PFOS ($p=0.182$). M65 was positively associated with PFHxS ($\beta=0.996\pm0.418$, $p=0.022$); PFOA ($\beta=1.460\pm0.720$, $p=0.044$) with a trend for PFOS ($p=0.149$). Thus, M30 and M65 were positively associated with every PFAS tested in at least one statistical model. No sex differences were noted. These data suggest that there may be a class effect for PFAS in the promotion of liver disease and caspase-3 mediated hepatocellular apoptosis. This effect was independent of sex.

In our data, three PFAS were associated with significantly reduced serum TNF α in both the univariate analysis (Supplementary Table) and multivariate final model [PFHxS ($\beta=-0.799\pm0.214$, $p<0.001$); PFOA ($\beta=-1.242\pm0.370$, $p=0.001$); and PFOS ($\beta=-0.704\pm0.196$, $p<0.001$)]. Likewise, two PFAS were associated with significantly reduced IL-8 in both the

Discussion

This study demonstrated that PFAA serum concentrations were positively associated with increased serologic biomarkers of hepatocyte death/apoptosis as well as altered inflammatory and adipokine disease biomarkers in humans (Table 3). This combination of increased apoptosis and decreased inflammation is a novel finding in this study. Unlike these PFAAs, other hepatotoxic chemicals have often been associated with liver necrosis and increased inflammation (Cave et al., 2011; Clair et al., 2018). The CK18 data extend the previously published associations between PFAS exposures and liver enzymes in the C8 Health Study and other cohorts (Darrow et al., 2016; Gallo et al., 2012; Gleason et al., 2015; Lin et al., 2010; Salihovic et al., 2018; Yamaguchi et al., 2013) by demonstrating apoptosis as a hepatocyte cell death mechanism. This is the major finding of the current study, and it is consistent with results from an animal exposure model (Kim et al., 2011). While liver biopsies and imaging are not available in the C8 Health Study, data from *in-vivo* and *in-vitro* models implicate that the observed apoptosis in the study (CK18 M30) elevations could be due to PFAA-associated fatty liver disease (Bodin et al., 2016; Kim et al., 2011; Kleszczynski et al., 2009). Indeed, CK18 M30 has been proposed as fatty liver biomarker (Feldstein et al., 2009; He et al., 2017). Moreover, in cohorts, PFAS exposures were associated with increased blood lipids possibly reflective of altered hepatic lipid metabolism and NAFLD (Frisbee et al., 2010; Geiger et al., 2014; Nelson et al., 2010; Steenland et al., 2009). While PFOA exposures were not associated with self-reported (and subsequently physician-validated) liver disease history in the C8 Health Study, there was increase in both ALT and in clinically abnormal ALT (Darrow et al., 2016). It is well-known that liver diseases (including NAFLD) may be subclinical in the absence of decompensated cirrhosis. No sex-differences were noted for either CK18 biomarker suggesting that sex may not modulate the apoptotic hepatic response to PFAS exposures. PFAS could initiate NAFLD via disrupting hepatic lipid metabolism via its pleiotropic effects on nuclear receptors, but more data are required.

The second important finding of the present study is the consistent and novel finding of an inverse relationship between serum PFAA levels and serum TNF α in humans. Liver disease and hepatocyte death are typically associated with increased hepatic inflammation and serum pro-inflammatory cytokines. However, PFAS including PFAAs are already known to be immunotoxic and to downregulate some aspects of the immune response. TNF α is a cell-signaling cytokine involved in acute phase reactions and chronic systemic inflammation. Reduced TNF α production has been documented following PFAA exposure in experimental systems (Corsini et al., 2014). PFAA serum concentrations were also associated with decreased lobular inflammation on liver biopsy in NAFLD patients (Rantakokko et al., 2015). However, mice fed a high fat diet and exposed to PFOA experienced liver cell inflammation (Tan et al., 2013). The potentially anti-inflammatory action of PFAAs is also supported by the inverse association between PFAS and IL-8 in our data. While positive associations were seen for PFAS with IFN γ and C3a, the roles of these pro-inflammatory molecules are less well established than TNF α in fatty liver diseases. Additionally, sex differences were observed between several pro-inflammatory biomarkers and PFAS exposures. More data are required on the potentially sex-dependent effects of PFAS on

histologic inflammation and liver disease progression. A third possible finding is the existence of sex differences for some markers, such as IL-8 and C3a and requires further evaluations in future, larger cohort studies.

NAFLD and other obesity-related diseases are typically associated with low adiponectin and high leptin. In our data, adiponectin was positively associated with PFHxS, PFOS, and PFNA exposures. While leptin usually moves discordantly to adiponectin, leptin was also positively associated with PFHxS and PFNA. Interestingly, adiponectin and leptin were also both increased in an animal PFOA exposure model (Du et al., 2018). The associations between PFAS and these adipokines have been investigated in several human developmental studies with conflicting results (Fleisch et al., 2017; Halldorsson et al., 2012; Minatoya et al., 2017). In the present study, sex differences were noted between the adipokines and PFAS exposures.

Only some patients with steatosis develop hepatic inflammation and nonalcoholic steatohepatitis (NASH), and only a subset of those progress to cirrhosis and hepatocellular carcinoma. Mechanisms implicated in the development of NASH include increased hepatocyte death, oxidative stress, and pro-inflammatory cytokines released by Kupffer cells, often in response to gut-derived lipopolysaccharide (LPS). Pollution exposures contributing to NASH include polychlorinated biphenyls (PCBs) or the volatile organic compound (VOC), vinyl chloride (VC); these appear to be second hits mediating the progression of diet-induced steatosis to NASH (Lang et al., 2018; Wahlang et al., 2014). These pollutants have been associated with increased serum biomarkers of hepatocyte necrosis (PCBs, VC); pro-inflammatory cytokines (PCBs, VC); and reduced antioxidants (VC) in exposed cohorts with liver disease (Cave et al., 2010; Clair et al., 2018). The terms, toxicant associated fatty liver disease (TAFLD) and steatohepatitis (TASH), have been proposed to describe the spectrum of fatty liver disease associated with chemical exposures (Wahlang et al., 2013). The current data support the presence of apoptosis in the absence of additional inflammation with PFAA exposures. When considering data from other studies that used rodent, fish and human cell line models to demonstrate the actual presence of fatty infiltration of liver cells following PFAS exposure and mechanisms thereof, (Fai Tse et al., 2016; Peng et al., 2013; Quist et al., 2015; Wang et al., 2013), our data possibly support a role for PFAAs in the initiation stage of NAFLD, or steatosis.

Neither liver biopsy nor imaging were obtained in the C8 Health Study, and it is important to address weaknesses of the biomarker approaches. The serum samples are derived from a cross-sectional study, and therefore do not address the long term follow-up needed to definitively document the development and progression of liver disease in a population. This cross-sectional design is a limitation because it only addresses associations. The serum samples investigated are randomly selected from a much larger cohort, and some random variation is a possible explanation for findings.

Likewise, unmeasured confounding can affect any single study. Furthermore, because this is a pilot study, no multiple comparison analyses were performed which is another drawback. However, in future confirmatory studies, adjustments for false discovery will be performed. An important weakness of using frozen serum is the possible effects of long term storage

and preexisting freeze-thaw cycles on results. In general, this concern would be most worrisome from a null bias perspective, but this study found hypothesized results for main outcomes (e.g., CK18 and TNF α) consistent with existing literature. Nevertheless, we wish to emphasize the scientific desirability of prospective studies on healthy humans with long term follow up and use of imaging and recently acquired sera for similar studies, and also note the strong ethical and logistical problems associated with that goal. Gene-environment interactions cannot be investigated using the C8 Health Study because only serum is available. Likewise, potential diet-PFAS interactions could not be evaluated in this study. In mice, short-term high fat diet feeding worsened PFAS-induced fatty liver disease and increased inflammation (Tan et al., 2013).

In summary, the most consistent findings of this study were associations between serum PFAA levels with increased CK18 M30, decreased TNF α , and increased adiponectin. Sex differences in the adipocytokine and C3a responses to PFAAs were observed. Based on the literature, the most plausible explanation for these findings is PFAA-induced toxicant associated fatty liver disease. We postulate that PFAAs disrupt hepatic lipid metabolism *via* nuclear receptor interactions to initiate steatosis with apoptosis while inhibiting Kupffer cell activation and upregulating adiponectin production. The latter two effects are expected to be protective. Thus, while PFAAs may initiate steatosis, they theoretically could also reduce the likelihood of subsequent progression of steatosis to steatohepatitis. However, based on the aforementioned limitations, this cross-sectional study must be considered preliminary with regards to liver disease progression. Nonetheless, these results demonstrate the need for more rigorously designed longitudinal studies of PFAS in the genesis and development of fatty liver disease. Because sex differences in disease mechanisms are also anticipated, sex as a biological variable must also be considered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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Abbreviations:

ADIP	adiponectin
ALT	alanine aminotransferase
AST	aspartate aminotransferase
C3a	cleaved complement 3

CAR	constitutive androstane receptor
CK18	cytokeratin 18
ERα	estrogen receptor α
eGFR	estimated glomerular filtration rate
FXR	farnesoid x receptor
HNF4α	hepatocyte nuclear factor 4 α
IFNγ	interferon γ
IL-6	interleukin 6
IL-8	interleukin 8
INS	insulin
LEP	leptin
LPS	lipopolysaccharide
LXR	liver X receptor
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
PAI-1	plasminogen activator inhibitor 1
PCBs	polychlorinated biphenyls
PFAAs	perfluoroalkyl acids
PFAS	perfluoroalkyl substances
PFOA	perfluorooctanoic acid
PFOS	perfluorooctane sulfonic acid
PFHxS	perfluorohexane sulfonic acid
PFNA	perfluorononanoic acid
PPARα and PPARγ	peroxisome proliferator activated receptors α and γ
PXR	pregnane X receptor
TAFLD	toxicant associated fatty liver disease
TASH	toxicant associated steatohepatitis
TNFα	tumor necrosis factor α
VC	vinyl chloride

VOC volatile organic compound**References**

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Highlights

- Serum levels of four perfluoroalkyl acids (PFAAs) and liver disease biomarkers were measured
- CK18 M30, an indicator of hepatocyte death, was positively associated with exposures to PFAAs
- Tumor necrosis factor α and interleukin 8 were inversely associated with exposures to PFAAs
- PFAAs exposures led to hepatic apoptosis but decreased inflammation, which is a novel finding

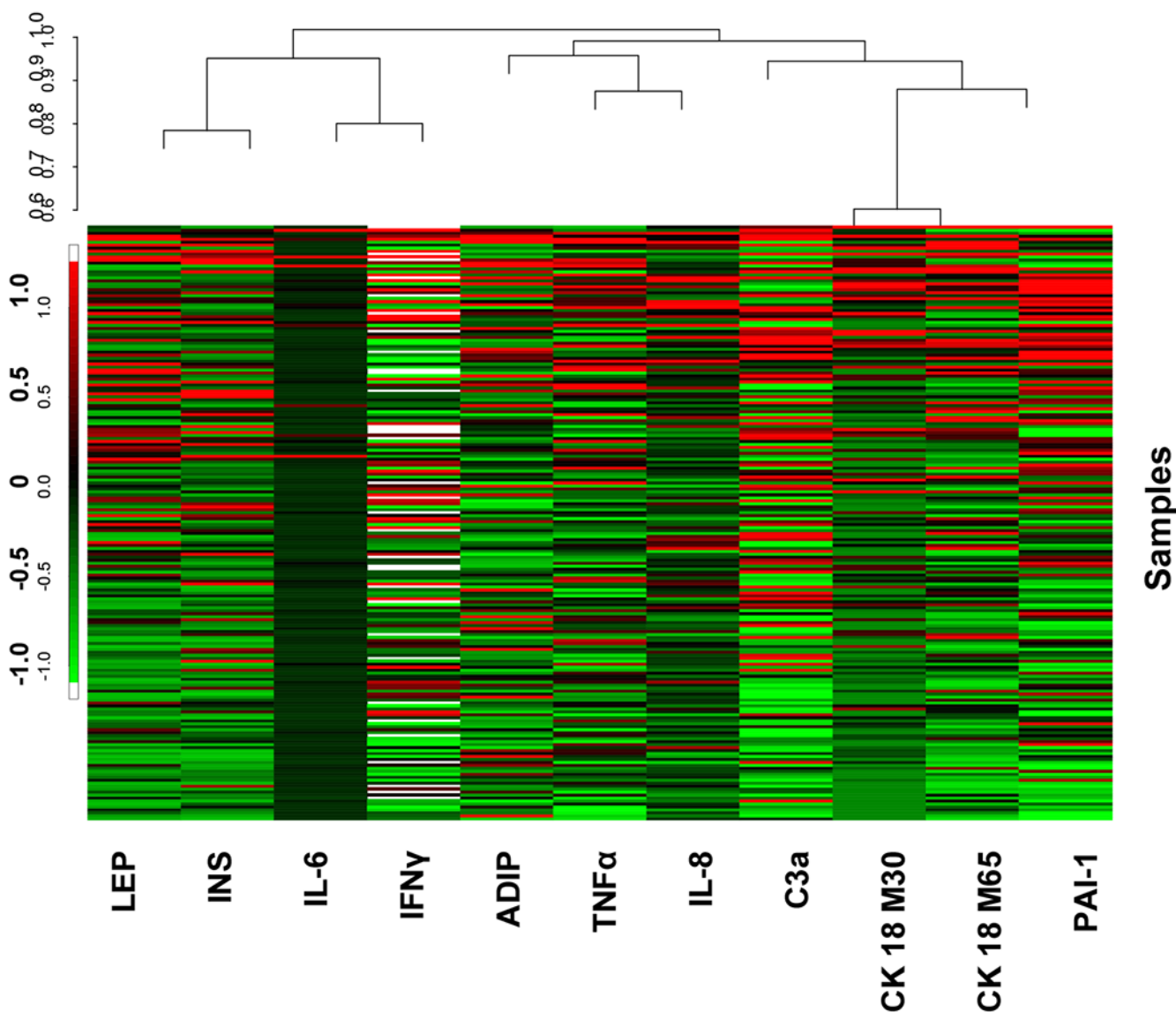


Figure 1. Disease Biomarker Heat Map

A heat map of the normalized logarithm of expression levels of biomarkers is demonstrated in colors that reflect expression levels (green represents low expression levels, red represents high expression levels). A color legend showing the normalized expression is on the left. The Venn diagram located in the upper part of the figure represents the exploratory hierarchical clustering analysis for associations among biomarkers based on their similarity, with the relative distance shown on the left axis (the smaller the closer).

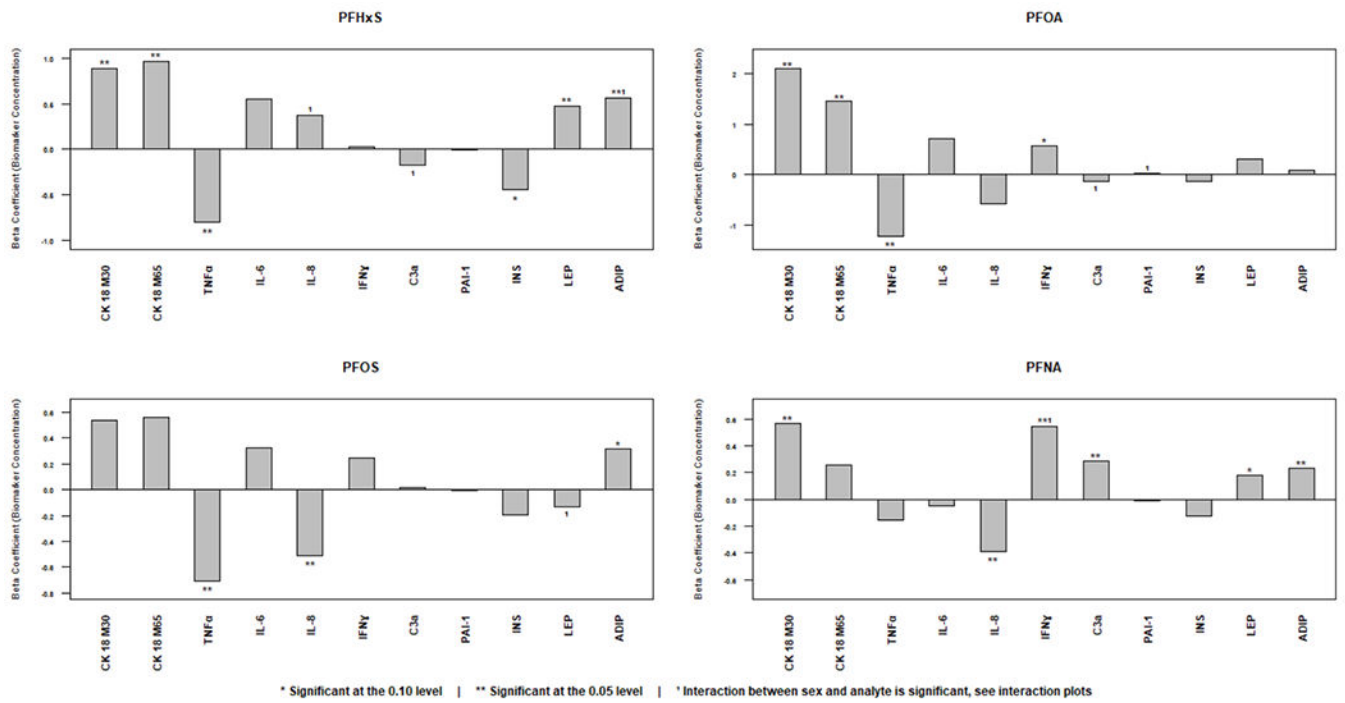


Figure 2. Significance of Biomarker Concentration in the Final Model

Adjusted beta coefficients are provided for each exposure and disease biomarker. These are graphical representations of the multivariate analysis results given in Table 2 (Final Model). Note that the range of the Y-axis varies between figures.

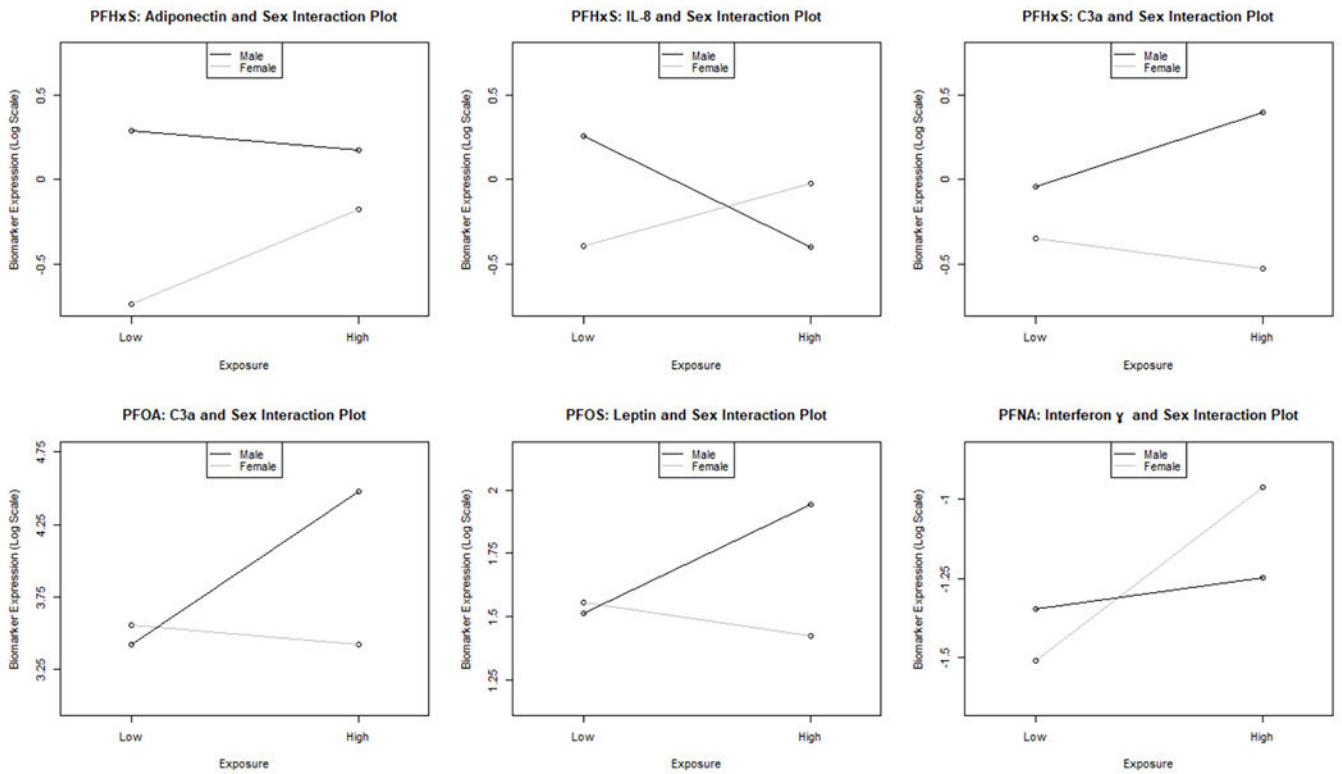


Figure 3. Significant Sex Differences in Biomarker Associations
 Significant relationships from the Interaction Model (Table 2) are depicted graphically. These figures are made using the coefficients from each respective linear model. The outcome variable (e.g., PFHxS) is continuous and log transformed, and the disease biomarker (e.g., adiponectin) is binary. One additional interaction was also statistically significant (PFOA and PAI-1) but is not displayed due to its small interaction coefficient estimate.

Table 1A:

Descriptive statistics for continuous factors

Variable	Overall		Male		Female		
	Mean (SD)	N	Mean (SD)	N	Mean (SD)	N	
Age (years)	55.3 (8.4)	200	55.4 (8.6)	92	55.2 (8.3)	108	
Weight (lbs)	181.1 (44.5)	200	200.6 (39.7)	92	164.4 (41.6)	108	
Height (inches)	66.9 (4.2)	200	70.6 (2.8)	92	63.7 (2.3)	108	
BMI	28.3 (6.0)	200	28.2 (4.7)	92	28.4 (7.0)	108	
ALT (U/L)	24.0 (12.5)	199	26.8 (14.5)	92	21.7 (9.87)	107	
AST (U/L)	22.8 (7.3)	199	23.0 (6.5)	92	22.50 (7.9)	107	
e-GFR (Calculated)	71.6 (15.5)	199	74.0 (15.9)	92	69.6 (14.9)	107	
Analyte (ng/mL)	PFHxS	4.2 (3.9)	200	5.1 (4.6)	92	3.4 (2.9)	108
	PFOA	94.6 (183.6)	200	97.0 (199.9)	92	92.6 (169.3)	108
	PFOS	26.9 (16.7)	200	29.1 (17.8)	92	25.0 (15.5)	108
	PFNA	1.6 (0.7)	200	1.6 (0.8)	92	1.6 (0.6)	108

* All available data used for analysis

Table 1B:

Descriptive statistics for categorical factors

Variable		N	Male ^I	Female ^I
			Frequency (%)	Frequency (%)
Current Alcohol User	Yes	78	43 (55.1)	35 (44.9)
	No	122	49 (40.2)	73 (59.8)
Average Alcohol Consumption	Not Classified	57	31 (54.4)	26 (45.6)
	1-3 Drinks per day	12	10 (83.3)	2 (16.7)
	A few drinks per week	23	14 (60.9)	9 (39.1)
	Less than one drink per month	48	22 (45.8)	26 (54.2)
	None	60	15 (25.0)	45 (75.0)
African American	Yes	38	22 (57.9)	16 (42.1)
	No	162	70 (43.2)	92 (56.8)
BMI Classification	Normal BMI (19.0 - 24.9)	81	34 (42.0)	47 (58.0)
	Overweight BMI (25.0 - 29.9)	55	25 (45.5)	30 (54.6)
	Obese BMI (≥ 30.0)	64	33 (51.6)	31 (48.4)

^INo missing values

Table 2:

Multivariate analysis, adjusting for e-GFR, alcohol consumption category, BMI, age and sex

Analyte	Biomarker ¹	Interaction Model ²			Final Model ³		
		Interaction Coefficient			Beta Coefficient (Analyte)		
		Estimate	SE	P Value	Estimate	SE	P Value
PFHxS	CK18 M30	-0.081	0.882	0.927	0.889	0.434	0.042
	CK18 M65	0.411	0.838	0.624	0.966	0.418	0.022
	TNF α	0.166	0.430	0.700	-0.799	0.214	<0.001
	IL-6	-0.022	1.009	0.983	0.551	0.487	0.260
	IL-8	-1.027	0.524	0.051	0.371	0.350	0.291
	Interferon γ	-0.261	0.383	0.498	0.032	0.191	0.868
	C3a	0.615	0.336	0.068	-0.176	0.234	0.453
	PAI-1	0.002	0.003	0.520	<-0.001	0.002	0.826
	Insulin	0.503	0.478	0.293	-0.442	0.231	0.057
	Leptin	0.172	0.338	0.611	0.471	0.168	0.006
	Adiponectin	-0.675	0.348	0.054	0.562	0.259	0.031
PFOA	CK18 M30	0.118	1.500	0.937	2.100	0.739	0.005
	CK18 M65	-0.736	1.445	0.611	1.460	0.720	0.044
	TNF α	0.479	0.743	0.520	-1.242	0.370	0.001
	IL-6	-1.259	1.735	0.469	0.718	0.839	0.393
	IL-8	-1.172	0.902	0.195	-0.593	0.441	0.180
	Interferon γ	0.162	0.651	0.804	0.580	0.325	0.076
	C3a	1.193	0.573	0.039	-0.136	0.400	0.733
	PAI-1	-0.010	0.005	0.059	0.003	0.004	0.469
	Insulin	1.212	0.827	0.177	-0.142	0.401	0.723
	Leptin	-0.074	0.592	0.900	0.310	0.294	0.292
	Adiponectin	0.046	0.606	0.940	0.079	0.310	0.800
PFOS	CK18 M30	0.198	0.811	0.807	0.536	0.400	0.182
	CK18 M65	-0.543	0.772	0.483	0.557	0.385	0.149
	TNF α	-0.115	0.394	0.771	-0.704	0.196	<0.001
	IL-6	-0.065	0.925	0.944	0.322	0.446	0.471
	IL-8	-0.511	0.477	0.586	-0.509	0.233	0.030
	Interferon γ	-0.533	0.349	0.128	0.245	0.175	0.163
	C3a	0.505	0.306	0.100	0.018	0.213	0.933
	PAI-1	0.002	0.003	0.390	<-0.001	0.001	0.673
	Insulin	0.409	0.440	0.354	-0.199	0.213	0.351
	Leptin	0.559	0.313	0.075	-0.131	0.221	0.554
Adiponectin	-0.277	0.318	0.385	0.318	0.163	0.053	
PFNA	CK18 M30	-0.203	0.531	0.702	0.567	0.262	0.031

Analyte	Biomarker ¹	Interaction Model ²			Final Model ³		
		Interaction Coefficient			Beta Coefficient (Analyte)		
		Estimate	SE	P Value	Estimate	SE	P Value
	CK18 M65	-0.383	0.511	0.454	0.254	0.255	0.320
	TNF α	0.279	0.267	0.297	-0.153	0.133	0.251
	IL-6	-0.166	0.611	0.786	-0.046	0.295	0.876
	IL-8	-0.184	0.314	0.557	-0.392	0.153	0.011
	Interferon γ	-0.447	0.222	0.046	0.546	0.156	0.001
	C3a	-0.074	0.200	0.710	0.282	0.101	0.006
	PAI-1	<0.001	0.002	0.861	<-0.001	0.001	0.928
	Insulin	0.345	0.290	0.236	-0.124	0.141	0.379
	Leptin	0.206	0.206	0.320	0.179	0.103	0.083
	Adiponectin	-0.2700	0.209	0.198	0.231	0.107	0.033

¹Units of biomarker: ADIP, LEP, INS, TNF α , IL-6, IL-8, IFN γ (pg/mL); M30, M65 (U/mL); C3a, PAI-1 (ng/mL)

²Interaction Model: Evaluates the interaction between biomarker concentration and sex

³Final Model: Interaction term only included if it was found to be significant from Interaction Model

Table 3:

Summary of statistically significant results by model

Exposure Variable	PFHxS		PFOA		PFOS		PFNA	
	Univariate <i>I</i>	Multivariate	Univariate <i>I</i>	Multivariate	Univariate <i>I</i>	Multivariate	Univariate <i>I</i>	Multivariate
Statistical Model								
Cytokeratin 18 M30	▲	▲	▲	▲	▲		▲	▲
Cytokeratin 18 M65		▲	▲	▲	▲		▲	
Tumor Necrosis Factor α	▼	▼	▼	▼	▼	▼		
Interleukin 6	▲							
Interleukin 8		*			▼	▼	▼	▼
Interferon γ				▲			▲	▲*
Cleaved complement 3		*	▲	*	▲		▲	▲
Plasminogen activator inhibitor 1				*	▼			
Insulin	▼	▼						
Leptin		▲				*	▲	▲
Adiponectin		▲*			▲	▲	▲	▲

I By Wilcoxon Ranked Sum Test

* Denotes significant sex difference, statistical significance at the 0.10 level

▲ – Indicates positive or negative direction of associations