

RESEARCH ARTICLE

Changes in hepato-renal gene expression in microminipigs following a single exposure to a mixture of perfluoroalkyl acids

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Abstract

It is evident that some perfluoroalkyl acids (PFAAs), a group of globally dispersed pollutants, have long biological half-lives in humans and farm animals. However, the effects of PFAAs in domestic animals have not been fully elucidated. The present study investigated how exposure to a single dose of a mixture of 10 PFAAs influenced hepatic and renal gene expression and histopathology, as well as plasma clinical biochemistry, in microminipigs (MMPigs) over 21 days. In animals treated with PFAAs, the mRNA expression of twelve genes related to fatty acid metabolism was upregulated in the kidney, while only few of these genes were induced in the liver. The expression of several kidney injury-associated genes such as, IGFBP1, IGFBP6, GCLC X2, GCLC X3, MSGT1, OLR1 was upregulated in the kidney. Interestingly, the expression of IGFBP-genes was differentially altered in the liver and kidney. Our findings thus identified hepato-renal gene expression changes in MMPigs that were associated with various molecular pathways including peroxisome proliferation, lipid metabolism, kidney injury, and apoptosis. Furthermore, serum HDL levels were significantly decreased following exposure to PFAAs, whereas no significant histopathological changes were detected, as compared to the vehicle group. Taken together, the present study provided the first indication that a single exposure to a mixture of PFAAs can produce changes in MMPig renal gene expression that were observed three weeks post exposure, suggesting that more attention should be paid to the kidney as a primary target organ of PFAAs.

Introduction

For several decades, perfluoroalkyl acids (PFAAs) have been used in a wide range of domestic and industrial applications because of their excellent physical and chemical properties. Consequently,

residues of numerous PFAAs have been detected at various concentrations in almost every environmental compartment around the world. For instance, they have been detected in human blood around the globe [1–7]. This ubiquitous distribution, environmental persistence, and potential human health concerns led to the listing of perfluorooctane sulfonate (PFOS) as a Persistent Organic Pollutant (POP) by the Stockholm Convention in 2009, within Annex B (Restriction). Guideline concentrations of PFOS and perfluorooctanoic acid (PFOA) have also been stipulated for food and drinking water [8,9] to restrict human consumption of these compounds.

PFAAs have been detected in livestock animals [10,11]. In beef cattle, PFOS mostly concentrates in the liver, followed by the kidney, which showed the longest elimination half-life of 385 days; the PFOS half-life in muscle was 165 days [12]. In pigs, the whole animal elimination half-lives are 634 days for PFOS and 236 days for PFOA [13]. These time periods are much longer than the commercial pig rearing period because these animals are normally slaughtered at approximately 180 days old within the pork industry. In chickens, the blood half-lives of PFOA and PFOS were 4.6 days and 125 days, respectively, and high concentrations of these chemicals were found in the kidney and liver, respectively [14]. Sex- and species-specific renal transporter proteins may play an important role in the renal clearance and resorption of PFAAs in humans and other animal species [15, 16]. Epidemiological studies further suggest that elevated human blood levels of PFOA and PFOS may be associated with kidney disease in adults and kidney dysfunction in children [17–18]. This suggests that the kidney is both important for the elimination of PFAAs and also a target organ for the effects of these compounds. Exposure experiments in animals reported that PFAAs elicited transcript signatures that included many known gene targets of peroxisome proliferator-activated receptor alpha (PPAR α), indicating effects on lipid metabolism [19]. Humans exposed to PFOA and PFOS showed altered blood expression of genes involved in cholesterol metabolism [20]. Nevertheless, the organ-specific effects of PFAAs on the transcriptome have not been elucidated for pigs.

Microminipigs (MMPigs) are the smallest pig species. These animals provide an attractive model for toxicological studies because they are easy to handle and cost-effective. We previously reported the blood elimination half-lives of 10 PFAAs, which are frequently detected in the environment, in MMPigs, in addition to their organ-specific accumulation patterns, following the administration of a single oral dose of mixed PFAAs [21]. However, the biological effects of exposure to PFAAs have not been evaluated in MMPigs. Moreover, we still lack an understanding of how PFAAs exposure regulates genes in farm animals. Therefore, the present study investigated the histopathological and transcription-level effects of a single PFAAs dose on MMPig hepatic and renal tissues, in addition to conducting analyses of their plasma clinical biochemistry. Previous animal exposure experiments have focused on hepatic, rather than renal, effects, even though substantial levels of PFAAs accumulate in the kidney, where they exhibit longer biological half-lives than those observed in the liver. To address this, we studied the expression of several genes associated with kidney injury, in addition to those involved in fatty acid metabolism.

Materials and methods

Animals and sample storage

The present study used frozen liver and kidney tissues from five sexually mature female MMPigs (Fuji Micra Inc., Shizuoka, Japan) aged 5.6–8.5 months and weighed 9–14 kg; these tissues were collected during a previous study [21]. In brief, the animals were separated into a control group ($n = 2$; C1 and C2) and an exposure group ($n = 3$; E1, E2, and E3). The animals were individually kept in stainless steel open-stall-type cages before and during the exposure. The exposure group was treated orally with a single gelatin capsule (100% pig) containing

3 mg kg⁻¹ body weight of a nominal mixture of each 10 PFAAs (30 mg kg⁻¹ body weight PFAAs) mixed with sugar powder. These compounds were perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), PFOA, perfluorononanoic acid (PFNA), perfluorononanoic acid (PFDA), perfluorodecanoic acid (PFUnDA), perfluorododecanoic acid (PFDoDA), and PFOS. Blood samples were collected before and after exposure, at 0, 1, 2, 4, 11, 15, and 21 days, and plasma samples were stored at -20°C until use. At 20 days, food was removed from the animals over 24 h before euthanasia under deep anesthesia with sodium pentobarbital, and the animals were necropsied to collect tissue samples. Details of the exposure protocol and the concentrations of PFAAs in blood and tissue samples have been reported previously [21]. This experiment was conducted according to the guidelines for animal experiments of the National Institute of Animal Health (NIAH, Tsukuba, Japan). The protocol was approved by the committee on the ethics of animal experiments of the NIAH (Protocol Number: 11-076).

Tissues (liver and kidney) were stored in RNAlater (Ambion Inc., Texas, USA) at -20°C for the evaluation of gene expression and were also fixed in 10% neutral buffered formalin and embedded in paraffin for histological examination. Paraffin sections were stained with hematoxylin and eosin.

Clinical biochemical measurements in plasma

The plasma levels of aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ -glutamyl transpeptidase (γ -GTP), total bilirubin (T-Bil), alanine aminotransferase (ALT), total protein (TP), blood urea nitrogen (BUN), albumin (Alb), uric acid (UA), creatinine (Cre), total cholesterol (T-Chol), triglycerides (TG), high-density lipoprotein cholesterol (HDL), lactate dehydrogenase (LDH) in plasma were investigated by SPOTCHEMTMEZ SP-4430 (ARKRAY, Inc., Kyoto, Japan). Low-density lipoprotein (LDL) was estimated by LDL (mg/dl) = (T-Chol) - (HDL + TG/5).

RNA isolation and analysis by reverse transcription-quantitative PCR

RNA was extracted from approximately 30 mg of each tissue using the Quick Gene RNA tissue kit S II (Kurabo Industries Ltd, Osaka, Japan) in accordance with the manufacturer's protocol and including DNase treatment.

Reverse transcription was carried out using total RNA (50 ng/ μ L) with PrimeScript RT Master Mix (Perfect Real Time) (Takara Bio Inc., Shiga, Japan) in accordance with the manufacturer's instructions. Real-time PCR reactions were carried out using SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio Inc., Shiga, Japan) using the protocol provided by the manufacturer. The real-time PCR conditions were 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. A melting curve analysis of the amplicons was then conducted at 95°C for 15sec, 55°C for 60 sec followed by 55–95°C at a heating rate of 0.5°C/sec. Genes were analyzed using a Mx3000P real-time qPCR system (Agilent Technologies, La Jolla, CA). The primer details are shown in Table 1 [22].

The PCR reactions were carried out using primers for cytochrome P4504A21 (CYP4A21), catalase (CAT), glycogen phosphorylase L (PYGL), stearoyl-CoA desaturase (SCD), fatty acid desaturase 1 (FADS1), fatty acid desaturase 2 (FADS2), acyl-CoA dehydrogenase (ACADM), enoyl-CoA hydratase (EHHADH), carnitine palmitoyltransferase 1A (CPT1A), acyl-CoA oxidase 1 (ACOX), peroxisome proliferator activated receptor alpha (PPAR α), and ATP binding cassette subfamily D member 3 (ABCD3). The primer sets for kidney injury biomarkers, including insulin like growth factor binding proteins (IGFBPs; 1, 2, 3, 4, 5, 6), glutamate-cysteine ligase catalytic subunit (GCLC), transcript variant X2 and X3, microsomal glutathione

Table 1. Primer sequences for real-time qPCR.

Gene Symbol	Forward Primer	Reverse Primer	Reference
CYP4A21	TTTTCCCGCTTGAGGAGTGC	ACTCGGTCTGTGTGTTGATGGA	This study
CAT	GTCCTGAGTCTCTGCATCAGGTT	GTTCATGTGCCTGTGTCCATCT	[22]
PYGL	GAAATTCTCCAGTGACCGAACAA	AGGTCGGAAGGCTCCATGT	This study
SCD	CTCTGGGCGTTTGCCTACTA	GGGGCAGTCGAGCTTTGTAA	[22]
FADS1	CCGCGACACAACACTACCACAA	CTTGGACTGGTACTCTATACCATGCTT	[22]
FADS2	CGCGACCTTGATTTAGTGCG	AGTTCTTGGTGCATCCTGG	[22]
ACADM	TGGCAATGAAAGTTGACCTAGCTA	TCGGCGACCAGAATCAATC	[22]
EHHADH	CCTCTGGAGCATCCTGGAAA	CAAGCCGAGAATGCCAACA	[22]
CPT1A	GGACATCCCGGAGGAGTGT	CACGTCGTCCGCCAGAA	[22]
ACOX	CTTTGTGCAGCGAGGACATC	CAAGGTGGCGAGGAACATG	[22]
PPAR α	GGCACTGAACATCGAATGTAGAAT	TGCAACCTTCACAGGCATGA	[22]
ABCD3	CGCGCTGGTGCCTCAT	TGGCGATGGCAAGACTGTT	[22]
IGFBP1	CACAGCAAACAGTGGGAGAC	CATTTGGGGTCCCTCTGAC	This study
IGFBP2	CCAGGAGTTCTGACATGCGT	CATCTCCAGCTGGGCATCTC	This study
IGFBP3	ACAAGAAAAAGCAGTGCCGC	CCGTACTTATCCACGCACCA	This study
IGFBP4	CAGCCCTCTGACAAGGACGAG	GCTCCGGTCTCGGATCTTGG	This study
IGFBP5	CTGTGACCGCAAGGGATTCT	GGCAGCTTCATCCCGTACTT	This study
IGFBP6	CCCTCGGGGGAGAATCCTAA	GAGGGAGTGGTAGAGGTCCC	This study
GCLC X2	TCCAGGTAACGTTCCAAGCC	TCAATGGGACAATGGGCAG	This study
GCLC X3	ACATCTACCACGCCGTCAG	GAGAGAGAACCAACCTCGTCG	This study
MGST1	CTCCTGCTCAGATCCACAATTC	ATAGGAGGCAAAGGCCATGAA	This study
OLR1	GCGGCAAACCTTTTCAGGTCC	AGCAGTTCTCCCGGCTTTT	This study
MFGE8	TTCGCCTTCTCCGGTGACTTC	TGGGGATCCTGGTCCAACAA	This study
LCN2	AAACCACGCTTTACGGGAGG	GACTTGGCAAAGCGGACAAA	This study
GAPDH	CCAGGTTGTGTCCTGTGACT	GCTTGACGAAGTGGTCGTTG	This study

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S-transferase 1 (MGST1), oxidized low density lipoprotein receptor 1 (OLR1), milk fat globule-EGF factor 8 protein (MFGE8), and lipocalin 2 (LCN2), were additionally used. The level of each gene transcript was normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct$ was the difference between the target gene cycle threshold [Ct]—GAPDH Ct of the exposure and control groups.

Statistics

The biochemical data were presented as the mean \pm standard error of the mean, and were analyzed by F-test and either Student’s t-test or Welch’s t-test. The statistical significance threshold was $p = 0.05$.

Results

Physiological status and the accumulation of PFAAs

No exposure-related clinical signs were observed during the 21-day study period [21]. The mean total concentration (\pm standard error) of PFAAs in the liver, blood and kidney was 75677 ± 22660 , 39307 ± 6700 and 23075 ± 3286 , respectively [21].

Clinical biochemistry in plasma

Biochemical analyses of plasma samples revealed significant differences in lipid metabolism. The HDL level was significantly lower in the exposure group, as compared to the control

group, at day 21 post exposure ($p < 0.05$, Fig 1). The T-Chol level in the exposure group tended to be higher than that of the control group on post exposure day 1 ($p < 0.1$), but no subsequent differences were observed during the study period (Fig 1). The ALP activity in the exposure group tended to be lower than that observed in the control group (S1 Fig), and this difference was significant at 15 days post exposure (exposure group: 370.7 ± 22.4 IU/L, control group: 676.5 ± 59.5 IU/L; $p < 0.05$). However, no differences were observed in the other plasma biochemistry parameters examined in the exposure and control groups (e. g. TG and LDL in Fig 1).

Histopathology

Histopathological examination revealed a slight decrease in the liver glycogen content in the exposure group, as compared to the control animals, but no other significant differences were detected in the liver or kidney tissues (Fig 2).

Metabolism-associated gene expression

The mRNA levels of metabolism-associated genes are shown in Fig 3. In the liver, the gene expression of CPT1A, PPAR α , and ABCD3 was increased (>1.5 -fold) in the exposed MMPigs, as compared to the control group (CPT1A: 2.3 ± 0.3 -fold; PPAR α : 1.7 ± 0.1 -fold; ABCD3: 1.8 ± 0.3 -fold) (mean \pm standard deviation); FADS2 expression was also increased by 1.5 ± 0.3 -fold. Interestingly, the mRNA levels of all metabolism-associated genes were

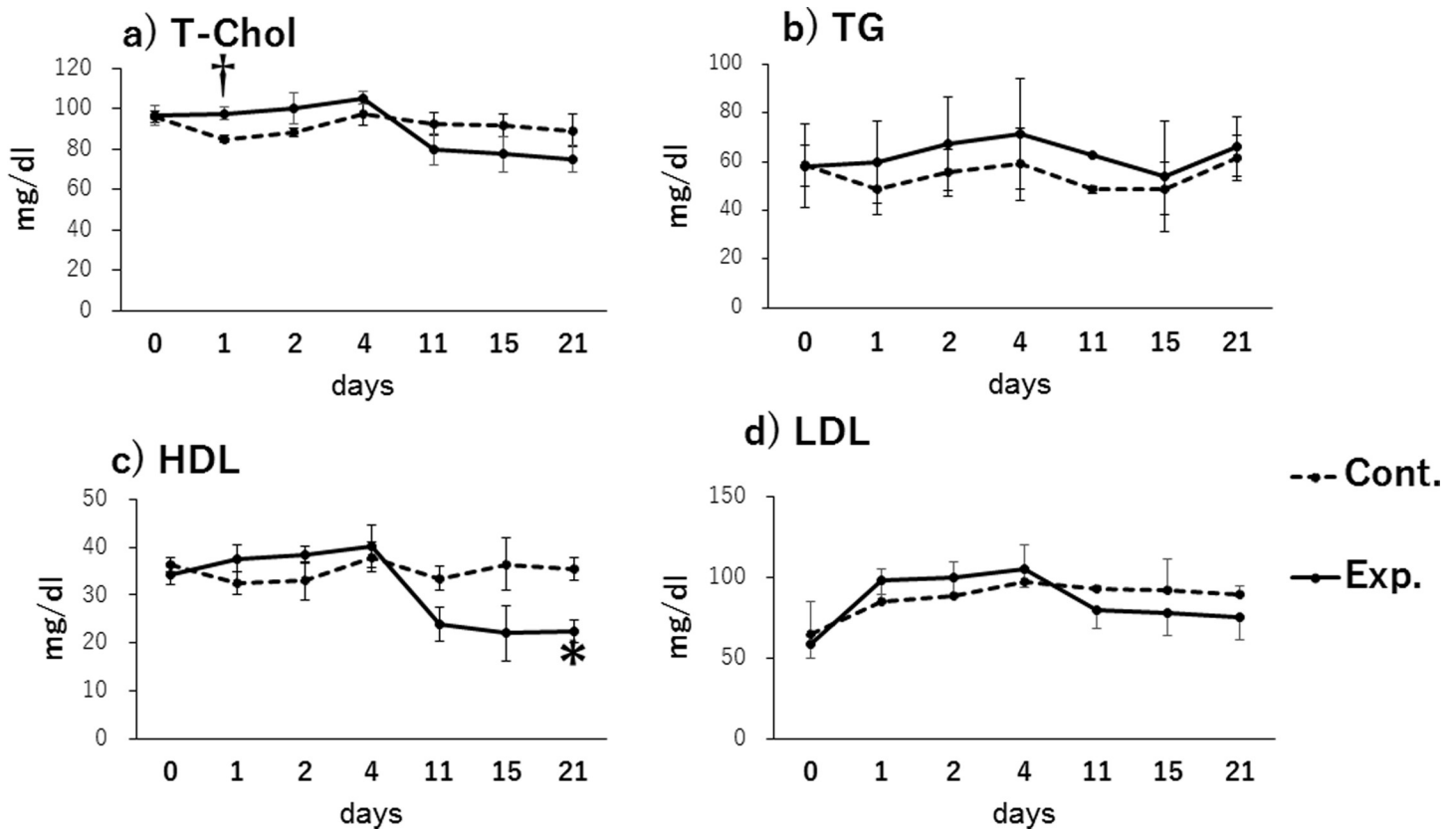


Fig 1. Levels of lipid metabolism markers in plasma. a) Total cholesterol (T-Chol); b) triglycerides (TG); c) high-density lipoprotein (HDL); d) Estimated Low-density lipoprotein (LDL). † $p < 0.1$, * $p < 0.05$ for the comparison between the control group (Cont.) and the exposure group (Exp.).

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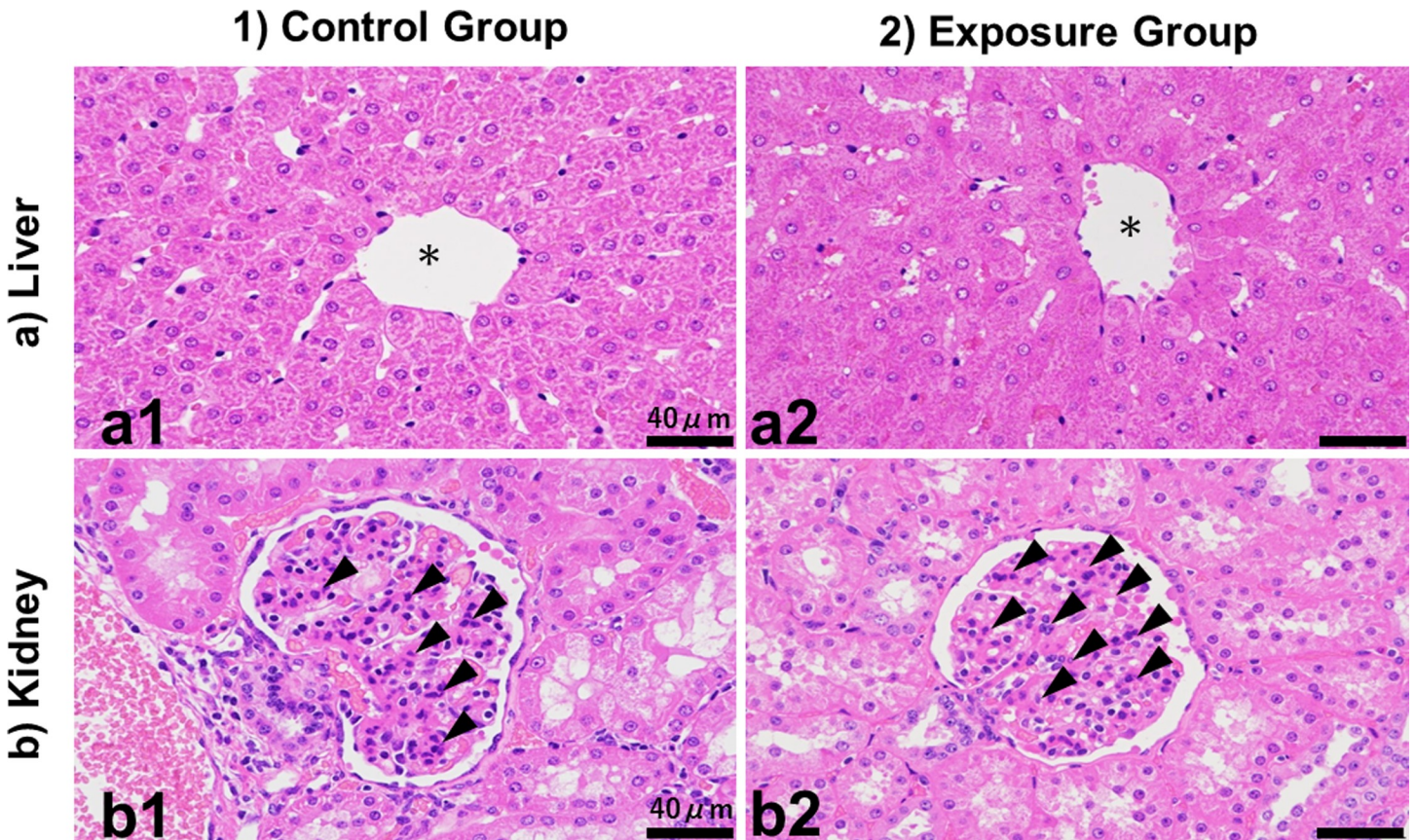


Fig 2. Results of histopathological examination. 1) Control group, 2) Exposure group. a) Sections of area around the central vein in liver. * shows a central vein, b): Sections of glomerulus in kidney. Arrows show mesangial cells and area. a2: Livers in exposure group have slightly low levels of glycogen granules compared to those in control group (a1). b1: and b2: Mesangial cells in glomerulus slightly increased in both control group and exposure group. Magnification x 400.

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upregulated in the kidneys of the exposure group, as compared to the control group: CYP4A21 (5.3 ± 3.1-fold), CAT (7.5 ± 1.4-fold), PYGL (1.7 ± 0.7-fold), SCD (8.4 ± 2.1-fold),

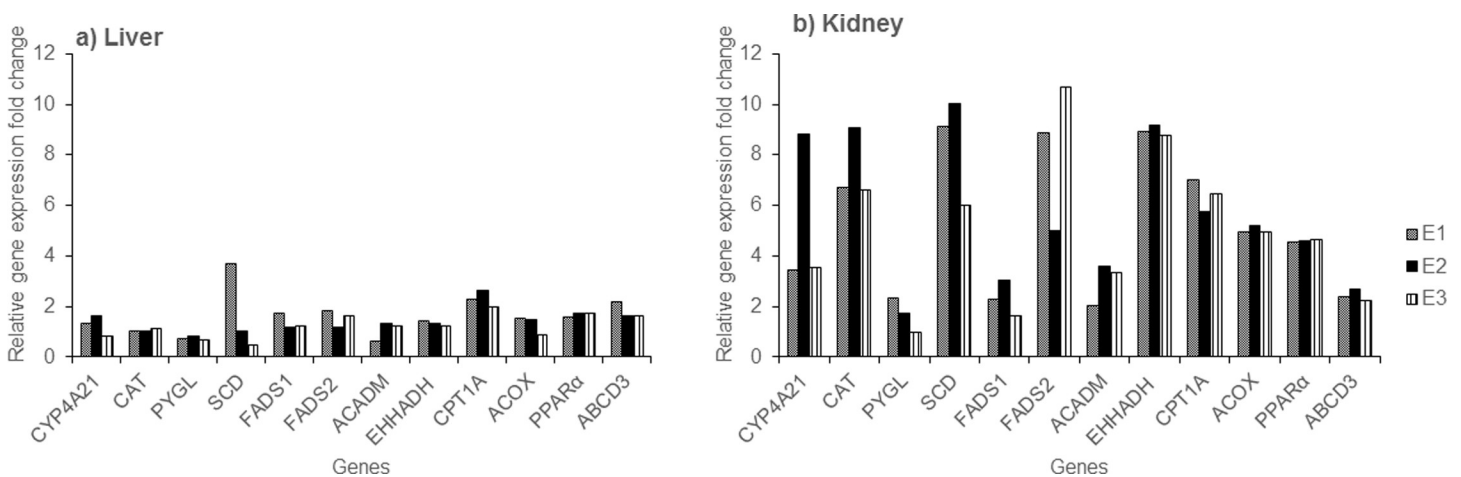


Fig 3. Relative mRNA expression in a) liver; and b) kidney tissues, expressed as fold change in the exposed group (n = 3; E1, E2, and E3). The genes analyzed were associated with fatty acid metabolism.

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FADS1 (2.3 ± 0.7 -fold), FADS2 (8.2 ± 2.9 -fold), ACADM (3.0 ± 0.8 -fold), EHHADH (8.9 ± 0.2 -fold), CPT1A (6.4 ± 0.6 -fold), ACOX (5.0 ± 0.2 -fold), PPAR α (4.6 ± 0.1 -fold), and ABCD3 (2.4 ± 0.2 -fold).

Kidney injury-associated gene expression

The mRNA levels of kidney injury-associated biomarkers are shown in Fig 4. In the liver, the expression of IGFBP1 was 2.3 ± 1.3 -fold higher in the exposure group, as compared to the control group. In contrast, the expression of IGFBP2 (0.5 ± 0.1 -fold), IGFBP3 (0.5 ± 0.1 -fold), IGFBP4 (0.3 ± 0.1 -fold), and IGFBP5 (0.4 ± 0.0 -fold) was lower in the exposure group than in the control group. Moreover, the expression of OLR1 (0.6 ± 0.2 -fold), MFGE8 (0.7 ± 0.1 -fold), and LCN2 (0.5 ± 0.2 -fold) also tended to be lower in exposed MMPigs, as compared to control animals.

In the kidney, IGFBP6 (4.1 ± 1.3 -fold) showed the greatest upregulation of the IGFBP family members; IGFBP1 (1.7 ± 0.8 -fold) tended to increase slightly, while IGFBP2 tended to decrease (0.4 ± 0.2 -fold), as compared to the control group. The expression levels of GCLC X2 (1.9 ± 0.6 -fold), GCLC X3 (1.5 ± 0.2 -fold), MSGT1 (1.5 ± 0.2 -fold), and OLR1 (1.9 ± 0.5 -fold) exhibited slight upregulation, as compared to the control group. No clear changes in the expression levels of IGFBP3, 4, 5, MFGE8, or LCN2 were observed in the exposure group, as compared to the control group (Fig 4).

Discussion

In the current study, we investigated changes in plasma clinical biochemistry, histopathology, and the expression of genes related to fat metabolism and kidney injury in MMPigs following the administration of a single dose of a mixture of PFAAs. We employed only 5 animals (control = 2 and treated = 3) to minimize the number of experimental animals. Although hyperplasia, vacuole formation, and atrophy have been reported in rats fed with PFOS [9], we only observed a slight decrease in the liver glycogen content in MMPigs (Fig 2), 21 days after exposure. In an earlier study, the liver glycogen content was reduced in pigs that had been fasted for 24 h or treated with clofibric acid (a potent peroxisome proliferator) for one week [22]. Hence, the minor effects on liver glycogen observed in the present study may have been influenced by exposure to PFAAs. The PFOS and PFOA concentrations in blood of MMPigs in this study were within the range of those reported in sera for occupationally exposed workers [1, 23].

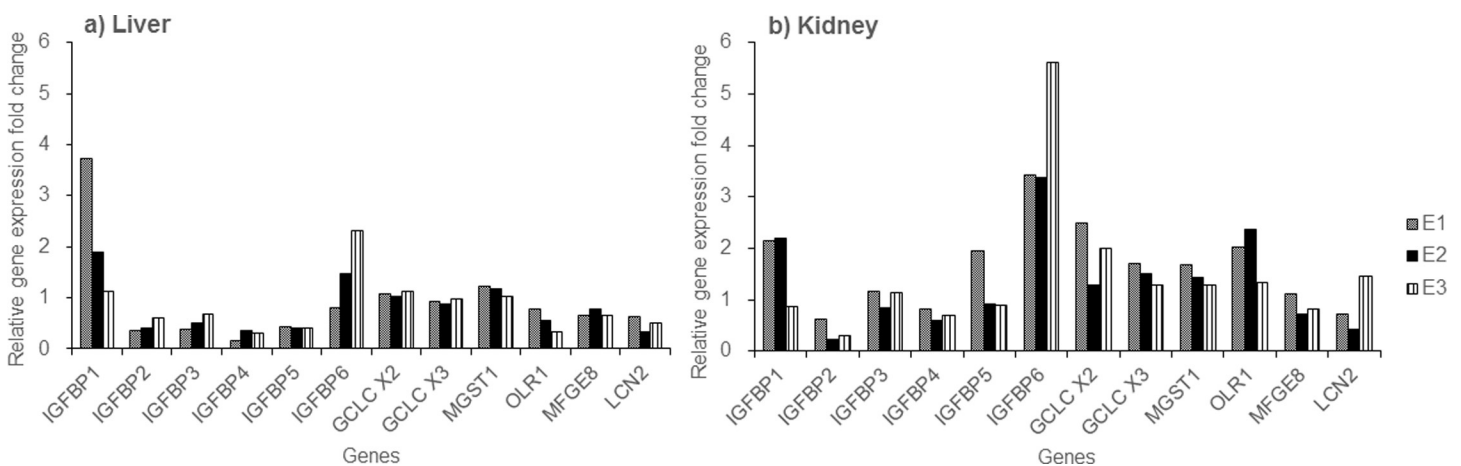


Fig 4. Relative mRNA expression of genes associated with kidney injury in a) liver and b) kidney, expressed as fold change in the exposed group (n = 3; E1, E2, and E3).

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Our observation of significantly lower plasma HDL concentrations in MMPigs exposed to PFAAs is consistent with previous sub-chronic PFOS exposure studies conducted in monkeys [24] and chickens [14]. The reason for the significant increase in total cholesterol level observed on day 1 post exposure is not known; however, a reduction relative to the controls was observed after 21 days. Seacat et al. [24] reported that although the serum levels of T-Chol and HDL varied during the exposure period, both of these levels were reduced by the end of the sub-chronic PFOS exposure period in monkeys, consistent with the present observation in MMPigs.

Exposure to PFAAs has been reported to interfere with a large number of PPAR α -dependent and -independent genes involved in fatty acid metabolism in rodents [25–27]. For instant, ACADM and CYP4A14 were upregulated in wild-type mice while ACOX1 and EHHADH were upregulated in both wild-type and PPAR α -null mice exposed to PFOS [26]. Nevertheless, gene-level effects on pigs exposed to PFAAs have not been documented. We noted that the average kidney/liver concentration ratio of PFAAs was 0.305 in these MMPigs [21]. However, the expression of genes related to fatty acid metabolism were highly upregulated in the kidney, as compared to the liver; this upregulation was disproportionate to the accumulation of PFAAs. These findings suggested that PPAR α and genes related to its oxidant pathway can be upregulated in the liver, and also in other important organs such as the kidneys, in pigs.

PPAR α can be upregulated in the pig liver by peroxisome proliferators, and also by fasting. The expression of genes responsible for PPAR α -mediated fatty acid metabolism such as CPT1A, ABCD3, ACOX, ACADM, and EHHADH was significantly or moderately increased in the pig liver in response to both fasting and clofibric acid treatment, while SCD expression was downregulated in fasted pigs, as compared to those receiving a normal diet [22]. In the present study, the expression of PPAR α , CPT1A and ABCD3 was upregulated in the liver and kidney of the exposure group, as compared to the control group. In addition, the kidney expression of ACOX, ACADM, and EHHADH also increased in the group exposed to PFAAs. Nevertheless, the duration of fasting may also have influenced these observations to some extent because food was removed from all animals 24 h before euthanasia, and the control group necropsy was carried out before that of the exposure group [21]. Moreover, we found an increased hepato-renal expression of FADS2 in the exposure group, as compared to the control group (Fig 3). In addition, CYP4A, CAT, PYGL, SCD, and FADS1 expression was upregulated in the kidney in the exposure group, as compared to the control group (Fig 3). It was previously reported that expression of CYP4A, CAT, PYGL, SCD, FADS1, and FADS2 genes was significantly increased by exposure to a peroxisome proliferator, but not by fasting, in pigs [22]. Collectively, these data suggest that PPAR α -mediated fatty acid metabolism was extensively elevated in MMPigs treated with PFAAs.

Epidemiological studies also suggested that kidney function may be associated with the blood levels of PFAAs [28]. As reported previously, higher levels of PFAAs in serum were linked with chronic kidney disease in domestic cats [29]. Hence, we analyzed twelve genes that were known to be associated with kidney function and injury. Expression of IGFBP1, IGFBP6, GCLC X2, GCLC X3 (GCLCs), MGST1, and OLR1 were upregulated in the kidney following exposure to PFAAs, as compared to the control group (Fig 4). The expression levels of IGFBPs, GCLCs, MGST1, and OLR1 genes are used as biomarkers of kidney injury [30–34]. Moreover, the growth hormone-insulin-like growth factor- (GH-IGF) axis plays a role in the maintenance of renal function and in the pathogenesis and progression of chronic kidney disease [32]. IGFBPs not only enhance the effects of IGFs, but also have IGF-independent activities; for instance, IGFBP1 and IGFBP6 levels increase in chronic renal failure [30]. The levels of IGFBP6 are particularly high in children with chronic renal failure, and correlate inversely with the glomerular filtration rate [35]. In addition, GCLCs, MGST1, and OLR1 can be

employed as markers of oxidative stress [31, 34, 36]. GCLC mediates the synthesis of glutathione S-transferase and thus plays an important role in the intracellular antioxidant defense system [37–38]. MGST1 is activated by oxidative stress and neutralizes lipid peroxides, as well as conjugating other reactive intermediates to glutathione [39]. Our present data suggest that the expression of these genes was upregulated due to oxidative stress, which occurred as a result of the PFAAs retained in the MMPigs, even 21 days post-exposure. LCN2, a neutrophil gelatinase-associated lipocalin, is a small glycosylated protein that is regarded as a biomarker of acute kidney injury and is thought to associate with chronic kidney disease progression in humans [40–43]. In addition, LCN2 is overexpressed by mesangial cells in the damaged kidney [40, 44]. Our histological analyses indicated that both control MMPig pigs and those exposed to PFAAs had mesangial lesions (Fig 2). However, this lesion might reflect age-related glomerulonephritis, as these animals were approximately 6 months old [45]. We did not observe any upregulation of LCN2 expression; however, as indicated above, upregulation of several other injury markers was observed in the absence of any histological evidence of kidney damage. Hence, our data suggested that transcriptional changes were observed in the kidney 3 weeks after a single exposure of MMPigs to a mixture of PFAAs, without any significant nephropathy.

The IGFs and IGFBPs play an important role in cell growth and differentiation [46]. The present study identified differential alterations in the expression of IGFBPs in the liver and kidney. The expression of IGFBP1 increased in both the liver (2.3 ± 1.3 -fold) and kidney (1.7 ± 0.8 -fold), IGFBP6 expression only increased in the kidney (4.1 ± 1.3 -fold), while reduced expression of IGFBP2 was observed in the liver (0.5-fold average) and kidney (< 0.5 -fold

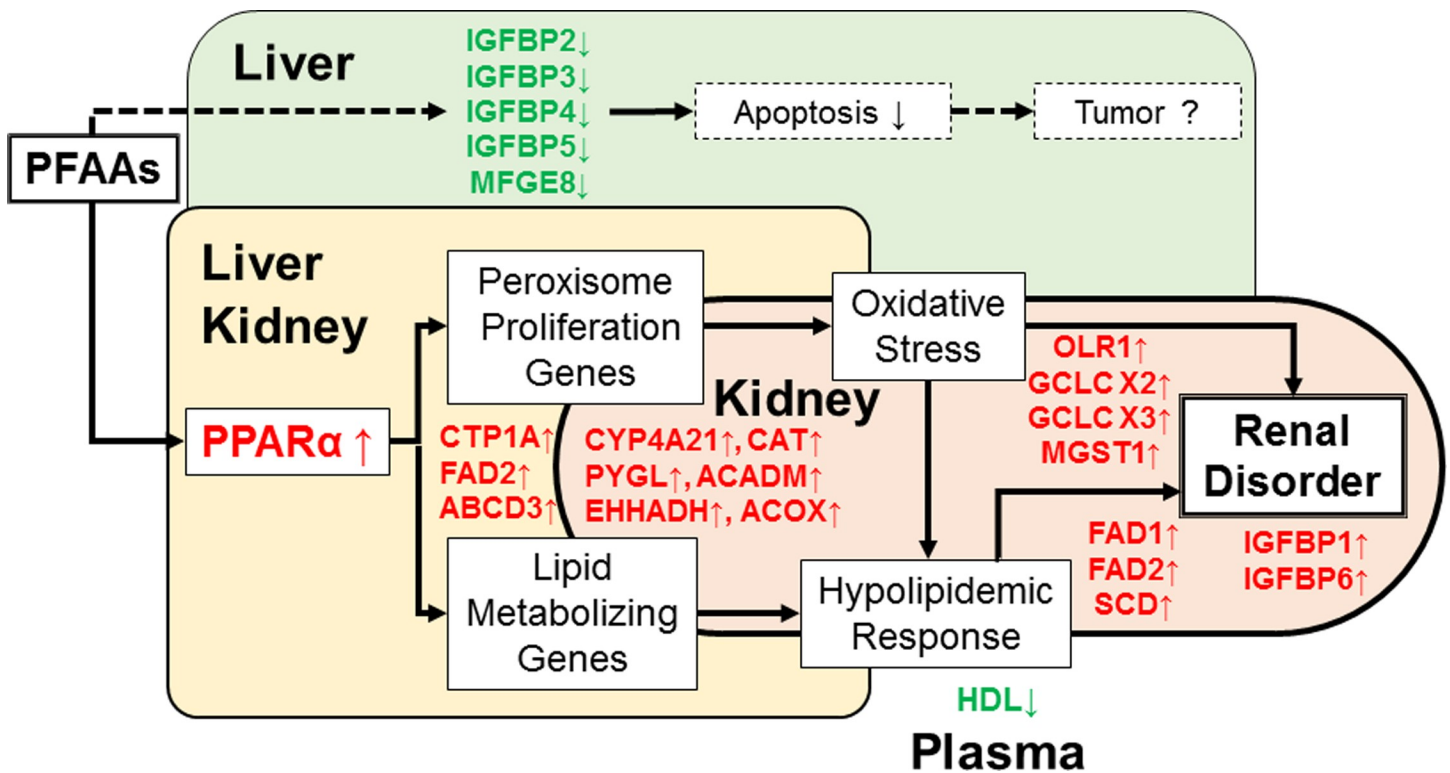


Fig 5. Hypothesized mode of action of PFAAs in MMPigs. The lines indicate major changes in gene expression and the downstream phenotypic responses noted in this study. The dashed lines indicate potential connections that remain to be verified.

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average), and the expression of IGFBP4 and IGFBP5 was decreased in the liver (< 0.5 -fold in average) (Fig 4). On the other hand, kidney expression of the oxidative stress biomarker, OLR1, was upregulated (1.9-fold average) while the serum level of HDL, which has antioxidant properties, was downregulated (Figs 1 and 4). Oxidative stress can lead to apoptosis, which is a process that inhibits tumor development. IGFBP2, 3, 4, and 5 are related to apoptosis, and IGFBP3 is known to have proapoptotic effects [30, 47]. We found that the expression of MFGE8, which is also related to apoptosis, and IGFBP3 was slightly decreased in the livers of the exposure group, as compared to the control group (MFGE8: 0.7 ± 0.1 -fold, IGFBP3: 0.5 ± 0.1 -fold, Fig 4). Taken together, these data suggest that exposure to PFAAs may link to the inhibition of apoptosis pathway in MMPigs.

Fig 5 shows the hypothesized mode of action of PFAAs in organs with special reference to the kidney of MMPigs, based on the observations made in this study. Our findings revealed that MMPigs exposed to PFAAs showed hepato-renal changes in the transcription of genes that were associated with cell proliferation, peroxisome proliferation, lipid metabolism, kidney injury, and apoptosis. Specifically, the present study revealed that a single exposure to a mixture of PFAAs was associated with changes in kidney gene expression at 21 days post exposure, in the absence of significant histological lesions. Nevertheless, further studies including microarray analysis are required to elucidate the molecular pathways involved in the potential adverse effects triggered by the exposure of large animals to PFAAs.

Supporting information

S1 Fig. Alkaline phosphatase (ALP) levels in plasma. † $p < 0.1$, * $p < 0.05$ for the comparison between the control group (Cont.) and exposure group (Exp.). (DOCX)

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References

1. Olsen GW, Burris JM, Mandel JH, Zobel LR. Serum perfluorooctane sulfonate and hepatic and lipid clinical chemistry tests in fluorochemical production employees. *J Occup Environ Med.* 1999; 41: 799–806. PMID: [10491796](https://pubmed.ncbi.nlm.nih.gov/10491796/)

2. Kannan K, Corsolini S, Falandysz J, Fillmann G, Kumar KS, Loganathan BG, et al. Perfluorooctanesulfonate and related fluorochemicals in human blood from several countries. *Environ Sci Technol*. 2004; 38: 4489–4495. PMID: [15461154](#)
3. Guruge KS, Taniyasu S, Yamashita N, Wijeratna S, Mohotti KM, Seneviratne HR, et al. Perfluorinated organic compounds in human blood serum and seminal plasma: a study of urban and rural tea worker populations in Sri Lanka. *J Environ Monit*. 2005; 7: 371–377. <https://doi.org/10.1039/b412532k> PMID: [15798805](#)
4. Nakayama S, Harada K, Inoue K, Sasaki K, Seery B, Saito N, et al. Distributions of perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) in Japan and their toxicities. *Environ Sci*. 2005; 12: 293–313. PMID: [16609670](#)
5. Calafat AM, Wong LY, Kuklennyk Z, Reidy JA, Needham LL. Polyfluoroalkyl chemicals in the U.S. population: data from the National Health and Nutrition Examination Survey (NHANES) 2003–2004 and comparisons with NHANES 1999–2000. *Environ Health Perspect*. 2007; 115: 1596–1602. <https://doi.org/10.1289/ehp.10598> PMID: [18007991](#)
6. Kärman A, Ericson I, van Bavel B, Darnerud PO, Aune M, Glynn A, et al. Exposure of perfluorinated chemicals through lactation: levels of matched human milk and serum and a temporal trend, 1996–2004, in Sweden. *Environ Health Perspect*. 2007; 115: 226–230. <https://doi.org/10.1289/ehp.9491> PMID: [17384769](#)
7. Eriksson U, Mueller JF, Toms LL, Hobson P, Kärman A. Temporal trends of PFSA, PFCA and selected precursors in Australian serum from 2002 to 2013. *Environ Pollut*. 2017; 220: 168–177. <https://doi.org/10.1016/j.envpol.2016.09.036> PMID: [27726977](#)
8. European Food Safety Authority (EFSA). Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts Scientific Opinion of the Panel on Contaminants in the Food chain. (Question No EFSA-Q-2004-163). *The EFSA Journal*. 2008; 653: 1–131.
9. U.S. EPA (United States Environmental Protection Agency). Drinking Water Health Advisory for Perfluorooctane Sulfonate (PFOS). 2016; docket 822-R-16-004.
10. Guruge KS, Manage PM, Yamanaka N, Miyazaki S, Taniyasu S, Yamashita N. Species-specific concentrations of perfluoroalkyl contaminants in farm and pet animals in Japan. *Chemosphere*. 2008; 73: S210–215. <https://doi.org/10.1016/j.chemosphere.2006.12.105> PMID: [18439642](#)
11. Kowalczyk J, Ehlers S, Oberhausen A, Tischer M, Fürst P, Schafft H, et al. Absorption, distribution, and milk secretion of the perfluoroalkyl acids PFBS, PFHxS, PFOS, and PFOA by dairy cows fed naturally contaminated feed. *J. Agric. Food Chem*. 2013; 61: 2903–2912. <https://doi.org/10.1021/jf304680j> PMID: [23441933](#)
12. Lupton SJ, Dearfield KL, Johnston JJ, Wagner S, Huwe JK. Perfluorooctane Sulfonate Plasma Half-Life Determination and Long-Term Tissue Distribution in Beef Cattle (*Bos taurus*). *J. Agric. Food Chem*. 2015; 63: 10988–10994. <https://doi.org/10.1021/acs.jafc.5b04565> PMID: [26684745](#)
13. Numata J, Kowalczyk J, Adolphs J, Ehlers S, Schafft H, Fuerst P, et al. Toxicokinetics of seven perfluoroalkyl sulfonic and carboxylic acids in pigs fed a contaminated diet. *J. Agric. Food Chem*. 2014; 62: 6861–6870. <https://doi.org/10.1021/jf405827u> PMID: [24892814](#)
14. Yoo H, Guruge KS, Yamanaka N, Sato C, Mikami O, Miyazaki S, et al. Depuration kinetics and tissue disposition of PFOA and PFOS in white leghorn chickens (*Gallus gallus*) administered by subcutaneous implantation. *Ecotoxicol. Environ. Saf*. 2009; 72: 26–36. <https://doi.org/10.1016/j.ecoenv.2007.09.007> PMID: [18006059](#)
15. Kudo N, Katakura M, Sato Y, Kawashima Y. Sex hormone-regulated renal transport of perfluorooctanoic acid. *Chem. Biol. Interact*. 2002; 139: 301–316. PMID: [11879818](#)
16. Andersen ME, Clewell HJ 3rd, Tan YM, Butenhoff JL, Olsen GW. Pharmacokinetic modeling of saturable, renal resorption of perfluoroalkylacids in monkeys-probing the determinants of long plasma half-lives. *Toxicology*. 2006; 227: 156–164. <https://doi.org/10.1016/j.tox.2006.08.004> PMID: [16978759](#)
17. Shankar A, Xiao J, Ducatman A. Perfluoroalkyl chemicals and chronic kidney disease in US adults. *Am. J. Epidemiol*. 2011; 174: 893–900. <https://doi.org/10.1093/aje/kwr171> PMID: [21873601](#)
18. Watkins DJ, Jossion J, Elston B, Bartell SM, Shin HM, Vieira VM, et al. Exposure to perfluoroalkyl acids and markers of kidney function among children and adolescents living near a chemical plant. *Environ. Health Perspect*. 2013; 121: 625–630. <https://doi.org/10.1289/ehp.1205838> PMID: [23482063](#)
19. Ren H, Vallanat B, Nelson DM, Yeung LW, Guruge KS, Lam PK, et al. Evidence for the involvement of xenobiotic-responsive nuclear receptors in transcriptional effects upon perfluoroalkyl acid exposure in diverse species. *Reprod. Toxicol*. 2009; 27: 266–277. <https://doi.org/10.1016/j.reprotox.2008.12.011> PMID: [19162173](#)
20. Fletcher T, Galloway TS, Melzer D, Holcroft P, Cipelli R, Pilling LC, et al. Associations between PFOA, PFOS and changes in the expression of genes involved in cholesterol metabolism in humans. *Environ. Int*. 2013; 57–58: 2–10. <https://doi.org/10.1016/j.envint.2013.03.008> PMID: [23624243](#)

21. Guruge KS, Noguchi M, Yoshioka K, Yamazaki E, Taniyasu S, Yoshioka M, et al. Microminipigs as a new experimental animal model for toxicological studies: comparative pharmacokinetics of perfluoroalkyl acids. *J. Appl. Toxicol.* 2016; 36: 68–75. <https://doi.org/10.1002/jat.3145> PMID: 25877231
22. Cheon Y, Nara TY, Band MR, Beever JE, Wallig MA, Nakamura MT. Induction of overlapping genes by fasting and a peroxisome proliferator in pigs: evidence of functional PPARalpha in nonproliferating species. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2005; 88: R1525–1535.
23. Olsen GW, Zobel LR. Assessment of lipid, hepatic, and thyroid parameters with serum perfluorooctanoate (PFOA) concentrations in fluorochemical production workers. *Int. Arch. Occup. Environ. Health.* 2007; 81: 231–246. <https://doi.org/10.1007/s00420-007-0213-0> PMID: 17605032
24. Seacat AM, Thomford PJ, Hansen KJ, Olsen GW, Case MT, Butenhoff JL. Subchronic toxicity studies on perfluorooctanesulfonate potassium salt in cynomolgus monkeys. *Toxicol Sci.* 2002; 68: 249–264. PMID: 12075127
25. Guruge KS, Yeung LW, Yamanaka N, Miyazaki S, Lam PK, Giesy JP, et al. Gene expression profiles in rat liver treated with perfluorooctanoic acid (PFOA). *Toxicol Sci.* 2006; 89: 93–107. <https://doi.org/10.1093/toxsci/kfj011> PMID: 16221955
26. Rosen MB, Das KP, Rooney J, Abbott B, Lau C, Corton JC. PPARα-independent transcriptional targets of perfluoroalkyl acids revealed by transcript profiling. *Toxicology.* 2017; 387: 95–107. <https://doi.org/10.1016/j.tox.2017.05.013> PMID: 28558994
27. Rosen MB, Schmid JR, Corton JC, Zehr RD, Das KP, Abbott BD, et al. Gene Expression Profiling in Wild-Type and PPARα-Null Mice Exposed to Perfluorooctane Sulfonate Reveals PPARα-Independent Effects. *PPAR Res.* 2010. 2010;pii: 794739.
28. Watkins DJ, Jossion J, Elston B, Bartell SM, Shin HM, Vieira VM, et al. Exposure to perfluoroalkyl acids and markers of kidney function among children and adolescents living near a chemical plant. *Environ Health Perspect.* 2013; 121: 625–630. <https://doi.org/10.1289/ehp.1205838> PMID: 23482063
29. Bost PC, Strynar MJ, Reiner JL, Zweigenbaum JA, Secoura PL, Lindstrom AB, et al. U.S. domestic cats as sentinels for perfluoroalkyl substances: Possible linkages with housing, obesity, and disease. *Environ Res.* 2016; 151: 145–153. <https://doi.org/10.1016/j.envres.2016.07.027> PMID: 27479711
30. Collett-Solberg PF, Cohen P. Genetics, chemistry, and function of the IGF/IGFBP system. *Endocrine.* 2000; 12: 121–136. <https://doi.org/10.1385/ENDO.12.2.121> PMID: 10905372
31. Kelly KJ, Wu P, Patterson CE, Temm C, Dominguez JH. LOX-1 and inflammation: a new mechanism for renal injury in obesity and diabetes. *Am J Physiol Renal Physiol.* 2008; 294: F1136–1145. <https://doi.org/10.1152/ajprenal.00396.2007> PMID: 18322020
32. Oh Y. The insulin-like growth factor system in chronic kidney disease: Pathophysiology and therapeutic opportunities. *Kidney Res Clin Pract.* 2012; 31: 26–37. <https://doi.org/10.1016/j.krcp.2011.12.005> PMID: 26889406
33. Fassett RG, Venuthurupalli SK, Gobe GC, Coombes JS, Cooper MA, Hoy WE. Biomarkers in chronic kidney disease: a review. *Kidney Int.* 2011; 80: 806–821. <https://doi.org/10.1038/ki.2011.198> PMID: 21697815
34. Sayanthoran S, Magana-Arachchi DN, Gunerathne L, Abeysekera T. Potential diagnostic biomarkers for chronic kidney disease of unknown etiology (CKDu) in Sri Lanka: a pilot study. *BMC Nephrol.* 2017; 18: 31. <https://doi.org/10.1186/s12882-017-0440-x> PMID: 28103909
35. Powell DR, Liu F, Baker BK, Hintz RL, Durham SK, Brewer ED, et al. Insulin-like growth factor-binding protein-6 levels are elevated in serum of children with chronic renal failure: a report of the Southwest Pediatric Nephrology Study Group. *J Clin Endocrinol Metab.* 1997; 82: 2978–2984. <https://doi.org/10.1210/jcem.82.9.4215> PMID: 9284730
36. Li D, Mehta JL. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. *Arterioscler Thromb Vasc Biol.* 2000; 20: 1116–1122. PMID: 10764682
37. Koide S, Kugiyama K, Sugiyama S, Nakamura S, Fukushima H, Honda O, et al. Association of polymorphism in glutamate-cysteine ligase catalytic subunit gene with coronary vasomotor dysfunction and myocardial infarction. *J Am Coll Cardiol.* 2003; 41: 539–545. PMID: 12598062
38. Lu SC. Glutathione synthesis. *Biochim Biophys Acta.* 2013; 1830: 3143–3153. <https://doi.org/10.1016/j.bbagen.2012.09.008> PMID: 22995213
39. Schaffert CS. Role of MGST1 in reactive intermediate-induced injury. *World J Gastroenterol.* 2011; 17: 2552–2557. <https://doi.org/10.3748/wjg.v17.i20.2552> PMID: 21633660
40. Schwartz N, Michaelson JS, Putterman C. Lipocalin-2, TWEAK, and other cytokines as urinary biomarkers for lupus nephritis. *Ann N Y Acad Sci.* 2007; 1109: 265–274. <https://doi.org/10.1196/annals.1398.032> PMID: 17785315

41. Mok CC. Biomarkers for lupus nephritis: a critical appraisal. *J Biomed Biotechnol.* 2010; 2010: 638413–638424 <https://doi.org/10.1155/2010/638413> PMID: [20414362](https://pubmed.ncbi.nlm.nih.gov/20414362/)
42. Viau A, El Karoui K, Laouari D, Burtin M, Nguyen C, Mori K, et al. Lipocalin 2 is essential for chronic kidney disease progression in mice and humans. *J Clin Invest.* 2010; 120: 4065–4076. <https://doi.org/10.1172/JCI42004> PMID: [20921623](https://pubmed.ncbi.nlm.nih.gov/20921623/)
43. Zhu L, Yu T, Qi X, Gao J, Huang K, He X, et al. Limited Link between Oxidative Stress and Ochratoxin A-Induced Renal Injury in an Acute Toxicity Rat Model. *Toxins.* 2016; 8: E373. <https://doi.org/10.3390/toxins8120373> PMID: [27983637](https://pubmed.ncbi.nlm.nih.gov/27983637/)
44. Qing X, Zavadil J, Crosby MB, Hogarth MP, Hahn BH, Mohan C, et al. Nephritogenic anti-DNA antibodies regulate gene expression in MRL/lpr mouse glomerular mesangial cells. *Arthritis Rheum.* 2006; 54: 2198–2210. <https://doi.org/10.1002/art.21934> PMID: [16804897](https://pubmed.ncbi.nlm.nih.gov/16804897/)
45. Shirota K, Koyama R, Nomura Y. Glomerulopathy in swine: microscopic lesions and IgG or C3 deposition in 100 pigs. *Nihon Juigaku Zasshi.* 1986; 48: 15–22. PMID: [3959370](https://pubmed.ncbi.nlm.nih.gov/3959370/)
46. Yu H, Mistry J, Nicar MJ, Khosravi MJ, Diamandis A, van Doorn J, et al. Insulin-like growth factors (IGF-I, free IGF-I and IGF-II) and insulin-like growth factor binding proteins (IGFBP-2, IGFBP-3, IGFBP-6, and ALS) in blood circulation. *J Clin Lab Anal.* 1999; 13: 166–172. PMID: [10414596](https://pubmed.ncbi.nlm.nih.gov/10414596/)
47. Nickerson T, Pollak M, Huynh H. Castration-induced apoptosis in the rat ventral prostate is associated with increased expression of genes encoding insulin-like growth factor binding proteins 2,3,4 and 5. *Endocrinology.* 1998; 139: 807–810. <https://doi.org/10.1210/endo.139.2.5912> PMID: [9449659](https://pubmed.ncbi.nlm.nih.gov/9449659/)