Efficacy, safety and novel targets in cardiovascular disease : advanced applications in APOE*3-Leiden.CETP mice

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Dose effects of ammonium perfluorooctanoate on lipoprotein metabolism in APOE*3-Leiden.CETP mice

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Abstract

Objectives: Epidemiological studies have reported positive associations between serum PFOA and total and non-high-density lipoprotein cholesterol (non-HDL-C) although the magnitude of effect of PFOA on cholesterol lacks consistency. The objectives of this study were to evaluate the effect of PFOA on plasma cholesterol and triglyceride metabolism at various plasma PFOA concentrations relevant to humans, and to elucidate the mechanisms using APOE*3-Leiden.CETP mice, a model with a human-like lipoprotein metabolism.

Methods and results: APOE*3-Leiden.CETP mice were fed a Western-type diet with PFOA (10, 300, 30 000 ng/g/d) for 4-6 weeks. PFOA exposure did not alter plasma lipids in the 10 and 300 ng/g/d dietary PFOA dose groups. At 30 000 ng/g/d, PFOA decreased plasma triglycerides (TG), total cholesterol (TC) and non-HDL-C, whereas HDL-C was increased. The plasma lipid alterations could be explained by decreased very low-density lipoprotein (VLDL) production and increased VLDL clearance by the liver through increased lipoprotein lipase activity. The concomitant increase in HDL-C was mediated by decreased cholesteryl ester transfer activity and changes in gene expression of proteins involved in HDL metabolism. Hepatic gene expression and pathway analysis confirmed the changes in lipoprotein metabolism that were mediated for a major part through activation of the peroxisome proliferator-activated receptor (PPAR)α.

Conclusions: Our data confirmed the findings from a phase 1 clinical trial in humans that demonstrated high serum or plasma PFOA levels resulted in lower cholesterol levels. The study findings do not show an increase in cholesterol at environmental or occupational levels of PFOA exposure, thereby indicating these findings are associative rather than causal.

Introduction

Salts of perfluorooctanoic acid (PFOA) were widely used as an emulsifier in the manufacture of fluoropolymers. Because PFOA is extremely stable (due to strong carbon-fluorine bond strength) and nonflammable, it cannot be readily degraded by strong acids, alkalis, or oxidizing agents; and as a result, it persists in the environment and is detected ubiquitously in humans and wildlife (1).

In general populations with ambient exposures or a community exposed to environmental levels of PFOA, several cross-sectional epidemiological studies have reported associations of serum PFOA with increased serum concentrations of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) in adults and children (2–8). However, the magnitude of effect on cholesterol appears to be less in more highly exposed occupational workers (9–16). In addition, there has been no reported increased risk for coronary artery disease incidence related to PFOA exposure in an exposed community (17) or in occupational cohorts when using internal referent comparisons (18,19).

Several studies have evaluated the effect of PFOA on plasma lipids, including mice (20–22), rats (20,23), and monkeys (24). In general, plasma lipids were either lowered or unchanged under these toxicological study conditions. These findings were consistent with one of the known toxicodynamic properties of PFOA in which it can activate nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα) and subsequently leads to increased fatty acid (FA) oxidation and an overall lowering of serum/plasma lipid levels (25–27). The activation of PPARα is the operative mechanism of fibrate drugs that reduce serum/plasma lipids in both laboratory animals and humans (28,29). In a phase 1 clinical trial study, PFOA was investigated as an antitumor agent due to its ability to inhibit PIM kinase activity (30). These patients received weekly ammonium PFOA doses that ranged between 50 and 1200 mg for 6 weeks. A reduction in plasma TC levels (LDL, not HDL-C) occurred at plasma PFOA concentrations between 420 and 565 μM (175 000–230 000 ng/mL). Albeit the exact mechanism was not fully elucidated, the finding reported by Convertino et al. (30) was consistent with the toxicodynamic property of PFOA described previously where hypolipidemic responses were observed in laboratory animals (20,22,23).

It is worth noting that, unlike humans, rodent lipoprotein metabolism is characterized by fast clearance of apoB-containing lipoproteins and the absence of cholesteryl ester transfer protein (CETP) resulting in a higher proportion of HDL-C relative to LDL-C (31). CETP is responsible for transferring cholesterol ester (CE) from HDL-C to the apoB-containing lipoproteins in exchange for triglycerides (TG). In contrast, human and nonhuman primates have a higher proportion of LDL-C relative to HDL-C due to the presence of CETP (31). Given the difference in lipid-handling that can hamper the extrapolation of rodent lipid data to human (31), we undertook this study to evaluate the effects of PFOA on lipid metabolism using the APOE*3-Leiden.CETP mouse model. This genetically engineered

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mouse model was designed to mimic the human lipoprotein metabolism with CETP expression and a delayed apoB clearance (31). It has been widely used to study the effect of drugs on atherosclerosis (32–35), cardiovascular safety (36,37), and lipid metabolism (38–40). Therefore, the objectives of the study reported herein are to (I) evaluate the effect of PFOA on plasma cholesterol at different PFOA concentrations that had been reported in human observational and experimental studies; and (II) elucidate the mechanism for the hypolipidemic responses with PFOA exposures, including hepatic gene expression and pathway analysis.

Materials and methods

Animals
Sixty-four male APOE*3-Leiden.CETP transgenic mice of 7–12 weeks (experiment 1) and 6–10 weeks (experiment 2) of age at the start of the experiment were used in this study. Mice were housed under standard conditions with a 12-h light-dark cycle and had free access to food and water. When fasting was required, only food was removed for the length specified by the study protocol. Body weight, food intakes, and clinical signs of behavior were monitored regularly during the study. Animal experiments were approved by the Institutional Animal Care and Use Committee of The Netherlands Organization for Applied Research (TNO) under registration number 3682. All procedures involving animals were conformed to Guide for the Care and Use of Laboratory Animals (41).

Human plasma samples
Plasma from healthy anonymized donors was obtained after their written informed consent from Sanquin blood bank (the Netherlands), in accordance with the Declaration of Helsinki.

Materials
PFOA ammonia salt (FC-143, lot 332) was provided by 3M Company (St Paul, Minnesota). It consisted of 77.6% linear and 22% branched (12.6% internal monomethyl (nonalpha), 9% isopropyl, 0.2% tert-butyl, 0.1% gem-dimethyl, and 0.1% alpha monomethyl). This test material, a white solid, was 97.99% pure and was stored at room temperature. All other chemicals were reagent-grade.

Study design
In this study, mice were fed a semisynthetic Western-type diet (WTD) for 4 weeks of a dietary run-in (acclimation) period prior to group allocation (**Figure 1**). The semisynthetic WTD consisted of 0.25% cholesterol (wt/wt), 1% corn oil (wt/wt), and 14% bovine fat (wt/wt; Hope Farms, Woerden, The Netherlands).

Time (weeks)	-4	-3	-2	-1	0	1	2	3	4	5	6
Experiment 1 n=8 mice per group — Control / 10 ng/kg/d / 300 ng/kg/d / 30 000 ng/kg/d	Run-in/acclimatization on a WTD				Matching; BW, FI; Plasma TC, TG, HDL-C; FFA, glycerol				BW, FI; Plasma TC, TG, HDL-C; FFA, glycerol, ALT	HL + LPL activity; EchoMRI; Collection of feces	BW, FI; Plasma TC, TG, HDL-C; FFA, glycerol, ALT; CETP activity; VLDL production; **Sacrifice**
Experiment 2 n=8 mice per group — Control / 10 ng/kg/d / 300 ng/kg/d / 30 000 ng/kg/d	Run-in/acclimatization on a WTD				Matching; BW, FI; Plasma TC, TG, HDL-C				BW, FI; Plasma TC, TG, HDL-C, ALT; VLDL clearance; **Sacrifice**; Hepatic lipids and pathology; Transcriptome analysis		

Figure 1 Study set-up. At the end of the 4-week run-in period (t=0 weeks), mice were randomized into 4 groups based on age, body weight, and baseline plasma TC, TG, and HDL-C levels measured at the end of the run-in period. Upon randomization, mice were either fed with WTD alone (control group) or WTD containing ammonium PFOA at 10, 300, or 30 000 ng/g/d (n=8 mice per dose group). Abbreviations: BW, body weight; CETP, cholesteryl ester transfer protein; FFA, free fatty acids; FI, food intake; HDL-C, high-density lipoprotein cholesterol; HL, hepatic lipase; LPL, lipoprotein lipase; MRI, magnetic resonance imaging; TC, total cholesterol; TG, triglycerides; VLDL, very-low-density lipoprotein; WTD, Western-type diet.

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For test material administration, ammonium PFOA was incorporated into this WTD at either 10, 300, or 30 000 ng/g/d (verification by liquid chromatography-tandem mass spectrometry [LC-MS/MS]). These dietary PFOA doses were chosen to achieve serum or plasma PFOA concentrations that had been reported in human observational or experimental studies. At the end of 4–6 weeks of dietary PFOA treatment at either 10, 300, or 30 000 ng/g/d, the mice were aimed to achieve comparable plasma PFOA levels found in the mid-Ohio river valley residents whose drinking water was contaminated with PFOA (ie, environmental exposure), fluorochemical production workers (ie, occupational exposure), or laboratory toxicological studies where reduced serum/plasma lipids were observed (ie, toxicological exposure), respectively.

Experiment 1 (6-week dietary treatment containing ammonium PFOA)

At the end of the 4-week run-in period (t=0 weeks), mice were randomized into 4 four groups based on age, body weight, and baseline plasma TC, TG, and HDL-C levels measured at the end of run-in period (n = 8 mice per dose group) after a 4-h fast by tail vein bleeding. Upon randomization, mice were fed with the WTD alone (control group) or WTD containing ammonium PFOA at either 10, 300, or 30 000 ng/g/d for 6 weeks (**Table 1**). Body weight and food intakes were monitored and recorded throughout the study for all mice. At the end of week 4, plasma TC, TG, free fatty acids (FFA), glycerol and alanine aminotransferase (ALT) were measured in blood samples from 4-h fasted mice collected by tail vein bleeding. At the end of week 5, hepatic lipase (HL) and lipoprotein lipase (LPL) activities were determined in plasma (after heparin injection). In addition, total fat mass was determined by EchoMRI image and 48–72 h feces were collected to measure excretion of bile acids and neutral sterols. At the end of week 6, in addition to plasma TC, TG, FFA, glycerol, and ALT determination, very low-density lipoprotein (VLDL) production was measured for all mice (**Figure 1**). Aliquots of plasma samples (approximately 20 μl) were collected from mice at week 0, 4, and 6 for PFOA concentration determination by LC-MS/MS. All mice were euthanized at the end of week 6, and perigonadal white adipose tissue (pWAT) and brown adipose tissue (BAT) were weighed.

Experiment 2 (4-week dietary treatment containing ammonium PFOA)

Similar to experiment 1, at the end of the 4-week run-in period (t=0 weeks), mice were randomized into 4 groups based on age, body weight, and baseline plasma TC, TG and HDL-C levels measured at the end of the run-in period (n=8 mice per dose group) after a 4-hour fast by tail vein bleeding. Upon randomization, mice were either fed with WTD alone (control group) or WTD containing ammonium PFOA at 10, 300, or 30 000 ng/g/d similar to experiment 1 described above (**Table 1**). Body weight and food intakes were monitored and recorded throughout the study for all mice. At the end of week 4, plasma TC, TG and ALT enzyme activity were measured for all mice in addition to VLDL-clearance evaluation. Aliquots of plasma samples (approximately 20 μl) were collected from mice at

Table 1 Dietary PFOA intake and mean plasma concentrations of PFOA

		Control	10 ng/g/d PFOA	300 ng/g/d PFOA	30000 ng/g/d PFOA
	[PFOA] in diet (ng/g)	<0.5	9 ± 1	273 ± 12	26 380 ± 683
Experiment 1	Dietary PFOA intake (ng/g bw/day)	0.0	10	291	30 238
	Plasma [PFOA], t=4 weeks (ng/mL)	<1.0	49 ± 4	1 350 ± 88	90 663 ± 8 867
	Plasma [PFOA], t=6 weeks (ng/mL)	5 ± 1	65 ± 7	1 524 ± 54	144 000 ± 13 406
Experiment 2	Dietary PFOA intake (ng/g bw/day)	0.0	10	298	29 476
	Plasma [PFOA], t=4 weeks (ng/mL)	<1.0	51 ± 5	1 395 ± 100	93 713 ± 4 827

Mice received a Western-type diet without or with 10, 300 or 30 000 ng/g/d PFOA, for 6 weeks (experiment 1) or 4 weeks (experiment 2). Dietary and plasma PFOA concentrations were measured by LC-MS/MS and dietary PFOA intake was calculated. Data are presented as mean ± SD. (n=8 mice per group, n=6 dietary samples).

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week 0 and 4 for PFOA concentration determination by LC-MS/MS. In experiment 2, all mice were euthanized on week 4 for liver-related analysis, including microscopic evaluation, detailed hepatic lipid profiling, and transcriptome analysis (**Figure 1**).

Determination of serum and dietary concentrations of PFOA

Dietary PFOA concentrations were determined by LC-MS/MS. Briefly, one gram of each dietary sample (sampled from top, middle, and bottom of the bag) were pulverized and followed by addition of 50% methanol in water (9 mL). After overnight incubation at room temperature on a roller shaker, samples were then sonicated for 15 min followed by centrifugation (2500 × g, 5 min). One hundred microliters of the top layer was transferred to a new tube which contained a fixed amount of the stable isotope-labeled internal standard (13C-PFOA), followed by the addition of 1 N formic acid (1 mL) and 100 µl of saturated ammonium sulfate. The solution was mixed by vortexing and then subjected to solid phase extraction (SPE) with Phenomenex Strata-X 3-ml SPE columns and LC-MS/MS according to the method described in Ehresman et al. (42). Serum PFOA concentrations were also determined by LC-MS/MS as described previously (42).

Plasma biochemical analysis

EDTA plasma samples were collected in week 0, 4, and 6 after a 4-h fast. Plasma TC, TG, FFA, and free glycerol were determined using enzymatic kits (TC: Roche/Hitachi, Mannheim,

Germany, catalogue# 11491458216 TG: Roche/Hitachi, catalogue# 11730711216, FFA: Wako diagnostics, Richmond, USA, catalogue# 434-91795 and 436-91995, free glycerol: Sigma-Aldrich, St. Louis, USA, catalogue# F6428) according to the manufacturer's protocols. HDL-C was measured after precipitation as described previously (35). Non-HDL-C levels were calculated by subtracting HDL-C from TC. The distribution of cholesterol over plasma lipoproteins was determined in group-wise pooled plasma by fast protein liquid chromatography (FPLC) (43). ALT enzymatic activity was measured by reflectance photometry using a Reflotron® Plus analyzer (Hoffman-La Roche, Mannheim, Germany).

CETP activity assay

Differences in CETP activity between APOE*3-Leiden.CETP mice and humans may affect the magnitude of the PFOA-induced increase of plasma HDL-C. Therefore, endogenous CETP activity was measured in human and mouse plasma with a fluorescent assay using donor liposomes enriched with nitrobenzoxadiazole-labeled cholesteryl esters (RB-CETP, Roar Biomedical, New York, New York) as described previously (44).

Hepatic VLDL-TG and VLDL-apoB production

All mice were fasted for 4h prior to the start of the experiment. During the experiment, mice were sedated with acepromazine-midazolam-fentanyl intraperitoneally [6.25 mg/kg acepromazine (Ceva Santé Animale B.V., Naaldwijk, The Netherlands), 6.25 mg/kg midazolam (Actavis, Baarn, The Netherlands), and 0.3125 mg/kg fentanyl (Bipharma B.V., Almere, The Netherlands)]. At t=0 min, blood was taken via tail bleeding and mice were intravenously (IV) injected with 100 μl phosphate-buffered saline (PBS) containing 20 μCi Trans[35]S-labeled methionine/cysteine (ICN Biomedicals, Irvine, California) to measure de novo apoB synthesis. After 30 min, the mice received a Triton WR1339 IV injection (500 mg/kg body weight), which inhibits LPL-mediated lipolysis, thereby blocking VLDL clearance. Blood samples were drawn at 0, 15, 30, 60, and 90 min after Triton WR1339 injection and used for determination of the plasma TG concentration. After 90 min, the animals were sacrificed by cervical dislocation and blood was collected by heart puncture for subsequent isolation of VLDL by density-gradient ultracentrifugation. ^{35}S-apoB was measured in the VLDL fraction after apoB-specific precipitation, and VLDL-apoB production rate was calculated as disintegration per minute (dpm)/h, as previously reported (40,45,46).

In vivo clearance of VLDL-like particles

All mice were fasted for 4h and injected in the tail vein with VLDL-like particles (80 nm) containing 3H-labeled FA (as glycerol tri[3H]-oleate, [3H]-TO) and 14C-labeled cholesteryl oleate (as [14C]-cholesteryl oleate, [14C]-CO). At t=2, 5, 10, and 15 min post-injection, blood was collected to determine the plasma decay of [3H]-TO and [14C]-CO. At 15 min, mice were euthanized by cervical dislocation and perfused with heparin 10 U/mL in ice-cold

PBS for 5 min. Organs (ie, liver, subcutaneous WAT [sWAT], BAT, spleen, lung and skeletal femoral muscle) were harvested and saponified overnight in 500 μl Solvable (Perkin-Elmer, Wellesley, Massachusetts) to determine [3H]-TO and [14C]-CO uptake. Retention of radioactivity in the saponified tissues was measured as % of the injected dose and the half-life of VLDL-[3H]-TO and [14C]-CO was calculated from the slope after linear fitting of semilogarithmic decay curves as described previously (40,45,46).

HL and lipoprotein lipase assay

Lipolytic activity of both LPL and HL was determined at t=5 weeks. To liberate LPL from the endothelium, 4-h fasted mice were injected IV with heparin (0.1 U/g body weight; Leo Pharmaceutical Products BV, Weesp, The Netherlands) and blood was collected after 20 min. Postheparin plasma was incubated with 0.2 ml of TG substrate mixture containing triolein (4.6 mg/mL) and [3H]-TO (2.5 μCi/mL) for 30 min at 37°C in the presence or absence of 1 M NaCl, which completely inhibits LPL activity, to estimate both the HL and LPL activity. The LPL activity was calculated as the fraction of total triacylglycerol hydrolase activity that was inhibited by the presence of 1 M NaCl and is expressed as the amount of FFAs released per hour per mL of plasma (45,47).

Liver histology

Liver samples (lobus sinister medialis hepatis) were collected, fixed in formalin and paraffin embedded, and sections (3 μm) were stained with hematoxylin and eosin (HE). The level of macrovesicular and microvesicular steatosis and hypertrophy relative to the total liver area was determined by a board-certified pathologist and expressed as the percentage of total liver area. Inflammation was scored by counting the number of aggregates of inflammatory cells per mm^2. Inflammatory aggregates, as marker of liver inflammation, are defined as a cluster, not a row, of ≥ 5 inflammatory cells (48).

Hepatic lipid analysis

Liver tissue samples of lobus sinister lateralis hepatis were homogenized in phosphate-buffered saline, and the protein content was measured using a Lowry protein assay. Lipids were extracted as described previously (49), separated by high-performance thin-layer chromatography on silica gel plates, stained and analyzed with ChemiDoc Touch Imaging System (Bio-Rad Laboratories Inc, Hercules, USA). TG, CE, and free cholesterol (FC) content were quantified using Image-lab version 5.2.1 software (Bio-Rad Laboratories Inc, Hercules, USA) and expressed per mg liver protein.

Excretion of fecal sterols and bile acids

Fecal excretion of neutral sterols and bile acids was determined in feces, collected during a 48- to 72-h time period at 2 consecutive time points at week 5, by gas chromatographic analysis as described previously (47).

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Hepatic gene expression and pathway analysis

Total RNA was extracted from the liver using the RNAbee isolation kit (Tel-Test Inc., Friendswood, USA, catalogue# CS-105B) and the Nucleospin RNA II mini spin kit (Macherey-Nagel GmbH & Co, Düren, Germany, catalogue# 740.955.50). Total RNA concentration was determined spectrophotometrically using Nanodrop 1000 (Isogen Life Science, De Meern, The Netherlands), and RNA quality was assessed using the 2100 Bioanalyzer (Agilent Technologies, Amstelveen, The Netherlands). The NEBNext Ultra Directional RNA Library Prep Kit for Illumina was used to process the samples according to the protocol "NEBNext Ultra Directional RNA Library Prep Kit for Illumina" (NEB #E7420S/L). Strand-specific messenger RNA sequencing libraries were generated and sequenced at GenomeScan (Leiden, The Netherlands). The libraries were multiplexed, clustered, and sequenced on an Illumina NextSeq 500 with a single-read 75-cycle sequencing protocol, 15 million reads per sample. The genome reference and annotation file Mus_Musculus.GRCm38 was used for analysis in FastA and GTF format. The reads were aligned to the reference sequence using Tophat 2.0.14 combined with Bowtie 2.1.0, and based on the mapped read locations and the gene annotation. HTSeq-count version 0.6.1p1 was used to count how often a read was mapped on the transcript region. Selected differentially expressed genes (DEGs) were used as an input for pathway and upstream regulator analysis through Ingenuity Pathway Analysis suite (www.ingenuity.com; Accessed November 2016). Calculated p-values <0.01 were used as threshold for significance in all analysis except for those in the 30 000 ng/g/d dose group for which we used the adjusted p-value of <0.05, the latter indicating a higher level of stringency. Gene set enrichment analysis was used to highlight the most important processes and pathways ranked based on their p-value of overlap (37,45,48).

Statistical analysis

Data are presented as means ± SD. A Kruskal–Wallis test was used to determine the significance of differences between the groups. Significance of differences of the individual groups with the control was calculated nonparametrically using a Mann–Whitney U-test and the rejection criteria were adjusted using a Bonferroni–Holm correction. IBM SPSS v24.0 was used for all analyses. p-Values ≤0.05 were considered statistically significant.

Results

Clinical observations and food intakes

No clinical signs of abnormal behavior were noted in any treatment group during the study. There was no PFOA treatment-related mortality observed in the study and PFOA dietary treatment did not appear to affect food intakes in the mice. Four mice died in experiment 1 due to anesthetic complications (2 mice in 10 ng/g/d, 1 mouse in 300 ng/g/d, and 1 mouse in 30 000 ng/g/d) and this was not considered to be treatment related.

Dietary and plasma concentrations of PFOA

Dietary PFOA concentrations were determined for each dose group and based on 5–6 samples per dose group. The mean dietary PFOA concentrations were 8.5 ± 0.8, 273.0 ± 11.6, and $26\,380 \pm 683.3$ ng/g for the 10, 300, and 30 000 ng/g/d dose groups, respectively, which represented $89.1 \pm 8.6\%$, $95.0 \pm 4.0\%$, and $91.8 \pm 2.4\%$ of the target doses (**Table 1**). Based on body weight and food intake, these dietary PFOA intakes are approximately 10, 300 and 30 000 ng/g/d, respectively (**Table 1**).

Mean plasma PFOA concentrations were measured in all animals from both experiments and are presented in **Table 1**. In both experiments, there were dose-dependent increases in plasma PFOA concentrations. After 4 weeks of dietary PFOA exposures, plasma PFOA concentrations in either experiment 1 or experiment 2 were very similar; they were at 49–51 ng/mL (from 10 ng/g dose group), 1350–1395 ng/mL (from 300 ng/g dose group), and 90 663–93 713 ng/mL (from 30 000 ng/g/d dose group). In experiment 1, plasma PFOA concentrations continued to increase through week 6 (**Table 1**).

Body weight and liver weight

In experiment 1, there was a statistically significant decrease in body weight of the 30 000 ng/g/d dose group mice at t=4 weeks (-10%, $p<0.01$) and at t=6 weeks (-18%, $p<0.001$) when compared with control. The body weight reduction was not observed in week 4 in experiment 2 (**Table 2**). There was also a statistically significant increase in liver weight from the 30 000 ng/g/d group in experiment 1 ($+150\%$, $p<0.001$) and in experiment 2 ($+190\%$, $p<0.001$), but not with the 10 or 300 ng/g/d dietary dose (**Table 2**).

The effect of PFOA on plasma lipids

PFOA decreased plasma TC and TG and increased HDL-C

PFOA exposure did not alter plasma lipids at 10 and 300 ng/g/d dietary PFOA dose groups, respectively (**Figure 2A–D**). In contrast, at 30 000 ng/g/d dietary dose group where plasma PFOA reached 144 000 ng/mL after 6 weeks of dietary PFOA exposure, it decreased plasma TG (-72%, $p<0.001$) (**Figure 2A**), TC (-33%, $p<0.001$) (**Figure 2B**), and non-HDL-C (-69%, $p<0.001$) (**Figure 2C**) relative to controls. There was a concomitant increase in HDL-C ($+130\%$, $p<0.001$) (**Figure 2D**). The lipoprotein profile at this high PFOA exposure

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Table 2 Body weight, food intake, liver weight, and plasma parameters of mice in experiment 1 and experiment 2

	Experiment 1 (t=4 weeks)			
	Control	**10 ng/g/d**	**300 ng/g/d**	**30 000 ng/g/d**
Body weight (gram)	28.9 ± 1.9	28.5 ± 2.5	28.5 ± 2.4	25.9 ± 1.1**
Food intake (gram/day/mouse)	2.8 ± 0.1	3.0 ± 0.4	2.8 ± 0.3	2.8 ± 0.3
Liver weight (gram)	NA	NA	NA	NA
Liver weight (% of body weight)	NA	NA	NA	NA
ALT (U/L)	145 ± 84	83 ± 27	217 ± 151	437 ± 112***
TC (mmol/L)	6.2 ± 0.9	6.8 ± 1.5	5.9 ± 1.1	4.3 ± 0.7**
HDL-C (mmol/L)	1.8 ± 0.3	1.5 ± 0.3	1.7 ± 0.3	3.6 ± 0.5***
non-HDL-C (mmol/L)	4.4 ± 0.9	5.3 ± 1.4	4.2 ± 1.1	0.7 ± 0.2***
TG (mmol/L)	1.7 ± 0.4	1.6 ± 0.3	1.2 ± 0.2	0.3 ± 0.0***
	Experiment 1 (t=6 weeks)			
	Control	**10 ng/g/d**	**300 ng/g/d**	**30 000 ng/g/d**
Body weight (gram)	30.2 ± 1.9	28.9 ± 3.0	29.6 ± 2.4	25.5 ± 1.0***
Food intake (gram/day/mouse)	3.0 ± 0.1	2.9 ± 0.4	3.0 ± 0.4	2.6 ± 0.3
Liver weight (gram)	1.4 ± 0.1	1.4 ± 0.3	1.5 ± 0.3	3.5 ± 0.2***
Liver weight (% of body weight)	4.5 ± 0.3	4.8 ± 0.5	5.2 ± 0.6	13.6 ± 0.1***
ALT (U/L)	95 ± 27	118 ± 70	123 ± 90	740 ± 161**
TC (mmol/L)	7.6 ± 1.3	6.9 ± 1.5	7.8 ± 2.7	5.1 ± 0.6***
HDL-C (mmol/L)	1.4 ± 0.3	1.5 ± 0.4	1.5 ± 0.2	3.2 ± 0.3***
non-HDL-C (mmol/L)	6.3 ± 1.4	5.4 ± 1.2	6.3 ± 2.7	2.0 ± 0.5***
TG (mmol/L)	1.8 ± 0.4	1.5 ± 0.4	1.5 ± 0.4	0.5 ± 0.0***
	Experiment 1 (t=4 weeks)			
	Control	**10 ng/g/d**	**300 ng/g/d**	**30 000 ng/g/d**
Body weight (gram)	27.6 ± 1.9	28.7 ± 2.5	28.3 ± 1.6	25.8 ± 1.9
Food intake (gram/day/mouse)	2.7 ± 0.2	3.0 ± 0.2	2.9 ± 0.1	2.6 ± 0.1
Liver weight (gram)	1.3 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	3.8 ± 0.3***
Liver weight (% of body weight)	4.9 ± 0.4	4.8 ± 0.4	5.2 ± 0.2	14.8 ± 0.6***
ALT (U/L)	178 ± 52	211 ± 118	533 ± 950	553 ± 81**
TC (mmol/L)	6.1 ± 0.7	6.4 ± 1.5	6.0 ± 1.1	4.9 ± 1.2
HDL-C (mmol/L)	2.4 ± 0.4	2.2 ± 0.3	2.0 ± 0.2	4.5 ± 0.9***
non-HDL-C (mmol/L)	3.7 ± 0.9	4.3 ± 1.5	4.0 ± 1.1	0.3 ± 0.2***
TG (mmol/L)	1.5 ± 0.5	1.6 ± 0.5	1.5 ± 0.2	0.4 ± 0.1***

Mice received a Western-type diet without or with 10, 300 or 30 000 ng/g/d PFOA for 6 weeks in experiment 1 or for 4 weeks in experiment 2. All parameters were measured individually, except for food intake which was measured per cage. Data are presented as mean ± SD (n=8 per group, n=5–7 per group for ALT, n=3–4 cages per group). **p<0.01, ***p<0.001 as compared with the control group. Abbreviations: ALT, alanine transaminase; HDL-C, high-density lipoprotein cholesterol; NA, not applicable; non-HDL-C, non-high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.
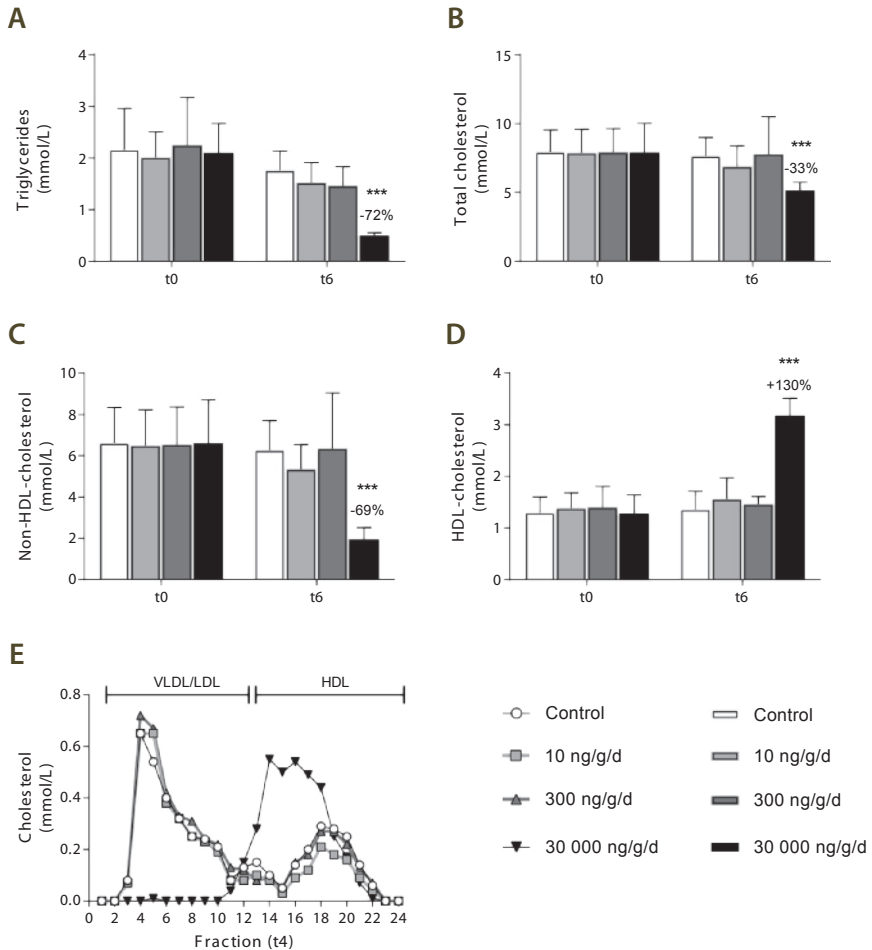
Figure 2 PFOA at low dose does not alter plasma lipids. Mice received a Western-type diet without or with 10, 300 or 30 000 ng/g/d PFOA. At baseline (t0) and after 6 weeks of exposure (t6), 4-h fasted blood was taken and plasma was assayed for TG (A), TC (B), non-HDL-C (C), and HDL-C (D). After 4 weeks of intervention, cholesterol distribution over lipoproteins was determined by FPLC in group-wise pooled plasma (E). Data are presented as means + SD (n=8 per group). ***p < .001 as compared with the control group. Abbreviations: FPLC, fast protein liquid chromatography; HDL-C, high-density lipoprotein cholesterol; VLDL/LDL, (very) low-density lipoprotein.

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exhibited a distribution pattern where most cholesterol was confined to large cholesterol ester-rich HDL (43) and almost none in VLDL-LDL (non-HDL) (**Figure 2E**).

PFOA decreased plasma FFA, plasma glycerol, pWAT, and BAT weight

After 6 weeks of PFOA exposure at the high dietary dose of 30 000 ng/g/d, total body fat mass (−66%, p<0.01), pWAT (−61%, p<0.01), and intrascapular BAT (−47%, p<0.001) were decreased compared with control (**Table 3**). These observations were accompanied by decreased plasma FFAs (−48%, <0.001) and glycerol levels (−42%, p<0.001) (**Table 3**), both of which are primarily derived from TG lipolysis in adipose tissue.

Table 3 PFOA decreases plasma FFAs, plasma glycerol and body fat

	Control	10 ng/g/d	300 ng/g/d	30 000 ng/g/d
Total body fat (gram)	3.5 ± 1.9	2.8 ± 0.9	3.3 ± 1.2	1.2 ± 0.3**
pWAT (gram)	0.8 ± 0.3	0.7 ± 0.3	0.6 ± 0.2	0.3 ± 0.1**
BAT (gram)	0.15 ± 0.03	0.13 ± 0.02	0.12 ± 0.02	0.08 ± 0.03**
FFA (mmol/L)	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	0.5 ± 0.1***
Free glycerol (mmol/L)	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.1 ± 0.0***

Mice received a Western-type diet without or with 10, 300 or 30 000 ng/g/d PFOA. In experiment 1, total body fat was measured by EchoMRI at week 5. At week 6, blood samples were taken after a 4-hour fast and plasma was assayed for FFAs and glycerol. At the end of experiment 1, BAT and pWAT were collected and weighted. Data are presented as mean ± SD (n=6-8 per group). ** P< 0.01, *** P<0.001 as compared to the control group. Abbreviations: pWAT, perigonadal white adipose tissue; BAT, brown adipose tissue; FFAs, free fatty acids; MRI, magnetic resonance imaging

PFOA increased plasma HDL-C levels by reducing CETP activity

Because high dose of PFOA at 30 000 ng/g/d did result in increased plasma HDL-C levels and HDL size (**Figure 2D and E**), we investigated whether this was caused by reduction of CETP activity. Indeed, PFOA significantly reduced CE transfer activity by 20% (p<0.01) in plasma of APOE*3-Leiden.CETP mice (**Figure 3A**). When we compared the CETP activity in plasma of the control APOE*3-Leiden.CETP mice with the activity in human plasma, we found a 7.5-fold higher activity in mice (**Figure 3B**). Taken together, the HDL-raising effect observed in this mouse model with PFOA treatment appears to be a consequence of the reduced CETP activity. This effect might be less pronounced in humans due to substantially lower CETP activity as illustrated in **Figure 3B**.

A

B

☐ Control ▦ 10 ng/g/d ▦ 300 ng/g/d ▪ 30000 ng/g/d
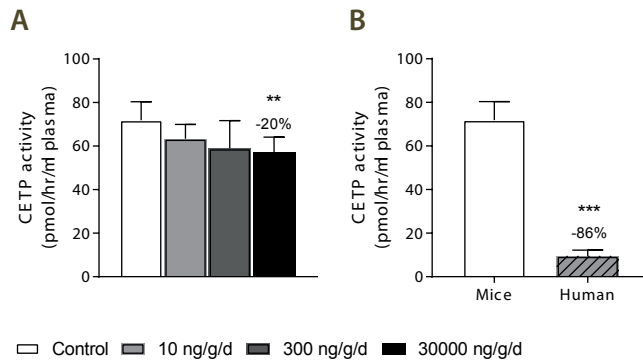
Figure 3 PFOA at high dose increases HDL-C by reducing CETP activity. Mice received a Western-type diet without or with PFOA in 3 different doses, 10, 300 and 30 000 ng/g/d. After 6 weeks of PFOA exposure in experiment 1, CETP activity was determined (A), and the activity in mice of the control group was compared with the activity in human plasma samples (B). Data are represented as mean + SD (n=6–8 mice per group and n=4 human plasma samples). **p<0.01 as compared with the control group and ***p<0.001 as compared with control APOE*3-Leiden.CETP mice. Abbreviations: CETP, cholesteryl ester transfer protein.

The effect of PFOA on hepatic lipoprotein and lipid metabolism

PFOA decreased hepatic VLDL-production rate

Because a decreased VLDL production may contribute to the overall TC- and TG-lowering effects, the VLDL production rate was determined in mice after 6 weeks of PFOA exposure. The VLDL-TG production rate was markedly decreased (−87%, p<0.001) by high dose PFOA exposure (30 000 ng/g/d dietary dose group) (**Figure 4A and B**) and a similar decrease was observed in the VLDL-apoB production rate (−86%, p<0.001) (**Figure 4C**). When normalized by apoB, the overall TG production rate per apoB was similar across all dose groups (**Figure 4D**), indicating that PFOA reduced the production rate of VLDL particles but did not alter the ratio between TG to apoB. These findings also provide an explanation for the marked reduction in total fat and pWAT and intrascapular BAT mass, and the correspondingly decreased plasma FFAs and glycerol levels through a reduced supply of VLDL-TG-derived FFAs for storage in adipose tissue. In contrast, VLDL production was not affected by either 10 or 300 ng/g/d dietary PFOA dose groups.

PFOA increased plasma VLDL clearance through enhanced LPL activity

Plasma VLDL-TG levels are defined by the balance between VLDL-TG production and VLDL-TG clearance, hence this part of the experiment evaluated the plasma clearance and tissue uptakes of [3H]-TO- and [14C]-CO-labeled VLDL-like particles after 4 weeks of PFOA exposure. At 10 or 300 ng/g/d dietary PFOA exposures, VLDL clearance was not affected
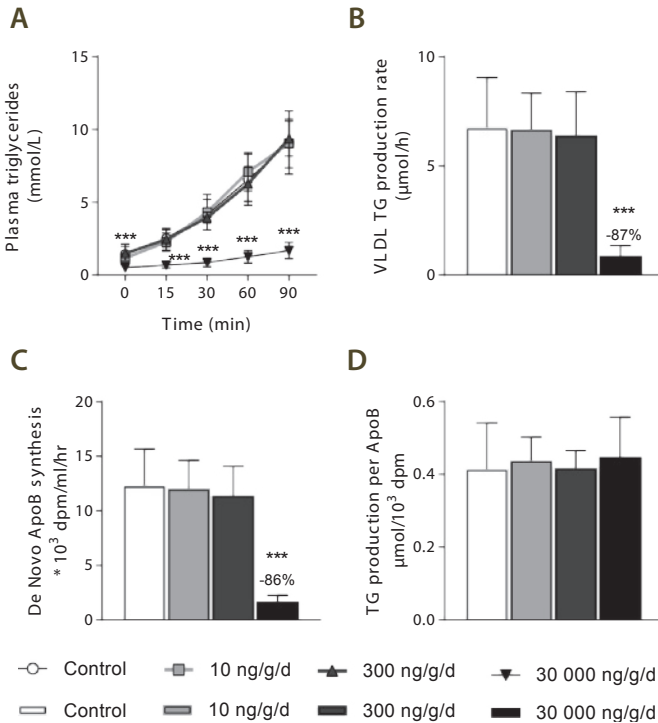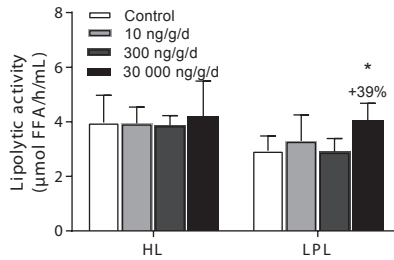
Figure 4 PFOA at high dose decreases VLDL-TG production and apoB synthesis. Mice received a Western-type diet without or with 10, 300 or 30 000 ng/g/d PFOA. After 6 weeks, 4-h fasted mice of experiment 1 were injected with Tran[35]S-label and Triton after which blood samples were drawn up to 90 min. Plasma VLDL-TGs (A) were plotted and used to calculate the rate of TG production (B) from the slope of the individual curves. Ninety minutes after Triton injection plasma was used to isolate VLDL by ultracentrifugation, and the rate of de novo ApoB synthesis was determined (C). The TG production per apoB was then calculated (D). Data are represented as mean ± SD (n=6–8 per group). ***p<0.001 as compared with the control group. Abbreviations: apoB, apolipoprotein B; TG, triglycerides; VLDL, very low-density lipoproteins.

(**Figure 5**). The uptake of [3H]-TO, representing FFAs and TGs, was increased by the high dose dietary PFOA treatment as indicated by decreased plasma [3H]-TO at t=15 min (−49%, p<0.05; **Figure 5A**), resulting in a significantly decreased half-life time (−30%, p<0.05; **Figure 5B**) relative to control. This effect was mainly due to increased [3H]-TO uptake by the liver (+54%, p<0.05), sWAT (+180%, p<0.01) and BAT (+90%, p<0.01; **Figure 5C**). The uptake of [14C]-CO, representing cholesteryl oleate, was not affected by PFOA exposure (**Figure 5D–F**).
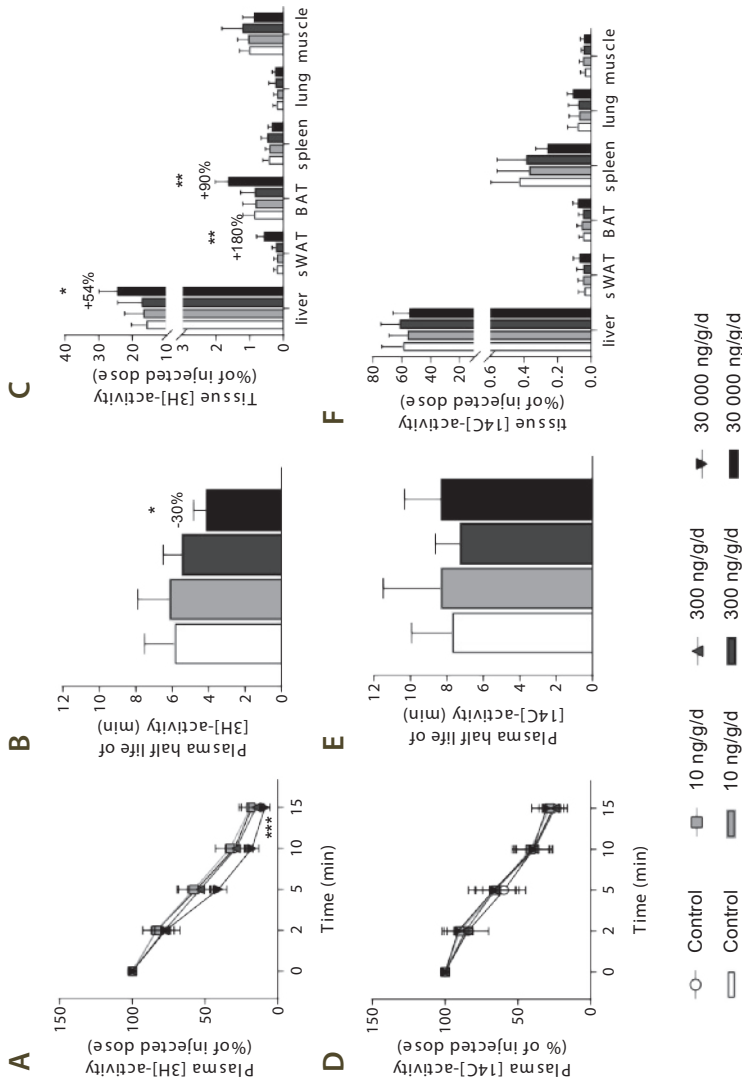
Figure 5 PFOA at high dose increases VLDL clearance mainly due to increased hepatic uptake. Mice received a Western-type diet without or with 10, 300 or 30 000 ng/g/d PFOA. After 4 weeks, 4-h fasted mice of experiment 2 were injected with glycerol tri[3H]-oleate ([3H]-TO) and [14C]-cholesteryl oleate ([14C]-CO)-labeled VLDL-like particles. [3H]-TO plasma decay was plotted (A) and used to calculate the rate of [3H]-TO uptake (B). Clearance of [3H]-TO in individual organs was determined (C). [14C]-CO plasma decay was plotted (D) and used to calculate the rate of [14C]-CO uptake (E). Clearance of [14C]-CO in individual organs was determined (F). Data are represented as mean ± SD (n=6–7 per group).*p<0.05, **p<0.01, and ***p<0.001 as compared with the control group. Abbreviations: BAT, brown adipose tissue; muscle, femoral muscle; sWAT, subcutaneous white adipose tissue; VLDL, very low-density lipoproteins.

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Because these data are consistent with increased lipolytic processing of VLDL particles, lipolytic activities (LPL and HL) were evaluated in experiment 1. After 5 weeks of PFOA exposure, mice were injected with heparin to liberate LPL from the endothelium, and LPL and HL activities were measured in plasma samples. HL activity was not affected by PFOA in any treatment group, but LPL activity was increased by the high PFOA dose at 30 000 ng/g/d (+39%, p <0.05) (**Figure 6**), providing an explanation for the increased FFA uptake by the liver and the adipose tissues. Collectively, these data indicate that high PFOA dose at 30 000 ng/g/d increased the hepatic uptake of FFAs, which together with the reduced VLDL production contributed to the decreased plasma lipid levels.

Figure 6 PFOA at high dose increases LPL activity. Mice received a Western-type diet without or with 10, 300 or 30 000 ng/g/d PFOA. After 5 weeks, 4-h fasted mice of experiment 1 were injected with heparin (0.1 U/g body weight) and postheparin plasma was collected. Plasma was incubated with a [3H]-TO-containing substrate mixture in the absence or presence of 1M NaCl, to estimate both the HL and LPL activity. Data are represented as mean + SD (n=5–8 per group). *p<0.05 as compared with the control group. Abbreviations: FFA, free fatty acids; HL, hepatic lipase; LPL, lipoprotein lipase; NaCl, sodium chloride.

PFOA resulted in hepatic hypertrophy without altering hepatic lipid contents

Consistent with previous findings in rodents (50,51) and monkeys (24), high dose PFOA at 30 000 ng/g/d resulted in increased ALT (**Table 2**) and hepatic hypertrophy, which was absent in control mice and was minimally observed in the 2 lower PFOA dose groups (**Figure 7A**). Because the decreased VLDL-TG production rate and increased VLDL clearance may be the result of changes in hepatic lipid metabolism, we evaluated liver pathology and measured hepatic lipid content and fecal excretion of bile acids and neutral sterols. Microscopically, macrovesicular steatosis was not observed with PFOA exposure (**Figure 7A**). While there was no microvesicular steatosis present in livers of control mice or in mice administered with the high PFOA dose group at 30 000 ng/g/d, a low amount (<5% of total liver area) of the liver area consisted of microvesicular steatosis (2.3 ± 5.2% and 4.7 ± 12.3%) in mice treated with 10 and 300 ng/g/d PFOA, respectively. The
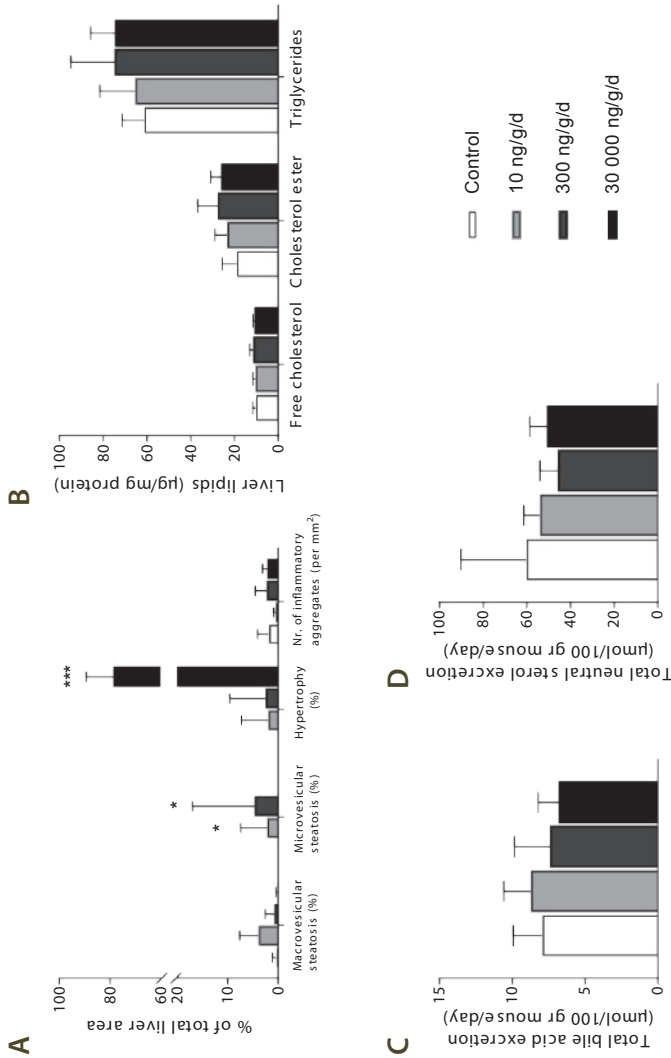
Figure 7 PFOA at high dose induces hepatic hypertrophy. Mice received a Western-type diet without or with 10, 300 or 30 000 ng/g/d PFOA. After 4 weeks of PFOA exposure in experiment 2, livers were histologically analyzed for macrovesicular and microvesicular steatosis, hypertrophy and the number of inflammatory aggregates per mm² (A), and hepatic lipid content per mg liver protein was measured (B). After 5 weeks of PFOA exposure in experiment 1, feces were collected per cage during a 48–72 h period at 2 consecutive time points, in which bile acid excretion (C) and neutral sterol excretion (D) were determined using gas chromatography. Data are represented as mean + SD (n=6–7 per group and n=6–8 collection points per group). *p <0.05 and ***p <0.001 as compared with the control group.

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number of inflammatory aggregates per mm^2, defined as a cluster of ≥5 infiltrating inflammatory cells (48), was not affected by any of the dietary PFOA groups.

Hepatic FC, CE, and TG contents were also not altered by any of the PFOA doses (**Figure 7B**). Similarly, no changes were observed in the excretion of bile acids (which are solely produced by the liver) and neutral sterols (**Figure 7C and D**). These data indicate that the decreased rate in VLDL-TG production is not explained by reduced availability of liver lipids for VLDL synthesis.

PFOA affected hepatic expression of genes involved in TG and cholesterol metabolism

To further investigate the mechanism by which PFOA affects lipid metabolism, gene expression of selected genes related to TG and cholesterol metabolism was determined in the liver as central organ in lipid metabolism. There were no significant changes in hepatic expression of lipid-related genes at the 2 lower PFOA dose groups. All significantly DEGs related to lipid metabolism in the liver mediated by the 30 000 ng/g/d PFOA dose group are depicted in **Table 4**. Although LPL expression in the liver is low as compared with heart, muscle, and adipose tissue, this PFOA exposure increased the expression of *Lpl* and decreased *Apoc3* expression, which is in line with the increased LPL activity and VLDL-TG clearance. Genes involved in FA/TG synthesis and VLDL assembly (*Scd3, Acss1, Scd2, Acsl3, Acsl4, Acsl5, Acsl1, Fasn, Acaca, Acss2,* and *Mttp*), FA β-oxidation (*Acss1, Ehhadh, Cpt1b, Acca1b, Acox1, Acsl3, Acsl4, Acsl5, Acsl1, Acca1a,* and *Acss2*), and uptake, transport, and binding of FAs (*Slc27a1, Fabp4, Cd36, Slc27a4,* and *Fabp1)* were increased. Collectively, these data indicate that PFOA in the liver increases FA oxidation, binding and activation, and mobilizes FA for TG synthesis and secretion as VLDL. Most likely, the latter 2 pathways are surpassed by increased FA uptake and transport. Additionally, the expression of *Apob* was decreased, which provides an explanation for the decreased VLDL-apoB formation.

PFOA affected hepatic expression of genes involved in HDL-C metabolism

High dose PFOA at 30 000 ng/g/d affected genes involved in HDL-C metabolism by decreasing the expression of *Apoa1* (the major gene in the formation of HDL), *Scarb1* (the principle gene in HDL-C clearance), and *Lipc* (plays a role in remodeling of HDL), and by increasing the expression of *Pltp* (which plays an important role in the remodeling of HDL by facilitating phospholipid transfer to HDL during its maturation from discoidal HDL into spherical HDL) (**Table 4**). Thus, together with the decreased CETP activity, changes in gene expression leading to reduced HDL-C uptake and formation of larger particles have contributed to the increased plasma HDL-C plasma levels and HDL size.

Table 4 The effect of 30 000 ng/g/d PFOA dose on hepatic expression of genes encoding proteins and transcription factors involved in TG and cholesterol metabolism

TG metabolism				ACS	Acsl4	2.3	<0.001
Protein	**Gene**	**Fold change**	**Padj**	ACS	Acsl5	2.3	<0.001
Lipolysis				ACS	Acsl1	2.1	<0.001
LPL	Lpl	4.3	<0.001	thiolase	Acaa1a	1.7	<0.001
HL	Lipc	0.5	<0.001	ACS	Acss2	1.7	0.010
APOC3	Apoc3	0.5	<0.001	ACO	Acox3	0.7	0.010
APOA5	Apoa5	0.4	<0.001	ACS	Acsm3	0.7	0.020
Fatty acid/TG synthesis				ACS	Acsm1	0.6	<0.001
SCD	Scd3	157.6	<0.001	ACS	Acsm5	0.6	0.002
ACS	Acss1	39.4	<0.001	PGC1alpha	Ppargc1a	0.6	0.007
SCD	Scd2	18.4	<0.001	ACS	Acsl6	0.1	0.006
ACS	Acsl3	2.5	<0.001	**FA uptake, transport, binding**			
ACS	Acsl4	2.3	<0.001	FATP	Slc27a1	9.8	<0.001
ACS	Acsl5	2.3	<0.001	FABP	Fabp4	8.6	<0.001
ACS	Acsl1	2.1	<0.001	CD36	Cd36	7.5	<0.001
FAS	Fasn	2.1	0.001	FATP	Slc27a4	2.8	<0.001
ACC	Acaca	1.9	0.001	FABP	Fabp1	1.7	0.010
ACS	Acss2	1.7	0.010	FATP	Slc27a5	0.6	<0.001
DGAT1	Dgat1	1.4	0.030	FABP	Fabp2	0.5	<0.001
ACS	Acsm3	0.7	0.020	**VLDL assemblage/formation**			
DGAT2	Dgat2	0.7	0.030	MTTP	Mttp	1.5	0.020
SREBP1a/c	Srebf1	0.7	0.040	APOB	Apob	0.8	0.050
ACS	Acsm1	0.6	<0.001	**Cholesterol metabolism**			
ACS	Acsm5	0.6	0.002	**Protein**	**Gene**	**Fold change**	**Padj**
ACS	Acsl6	0.1	0.006	**HDL maturation**			
Beta oxidation				LCAT	Lcat	0.6	<0.001
ACS	Acss1	39.4	<0.001	**HDL formation and remodeling/ destabilisation**			
bifunctional enzyme	Ehhadh	34.3	<0.001	APOA1	Apoa1	0.2	<0.001
CPT1	Cpt1b	4.6	<0.001	PLPT	Plpt	3.2	<0.001
thiolase	Acaa1b	3.0	<0.001	HL	Lipc	0.5	<0.001
ACO	Acox1	2.6	<0.001	**HDL uptake**			
ACS	Acsl3	2.5	<0.001	SRB1	Scarb1	0.5	<0.001

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Table 4 Continued

Synthesis				Metabolism			
HMGCS	*Hmgcs1*	2.8	<0.001	BSEP	*Abcb11*	0.5	<0.001
HMG CoA synthase	*Hmgcs2*	1.7	0.001	NTCP	*Slc10a1*	0.3	<0.001
				CYP7A	*Cyp7a1*	0.2	<0.001
Storage				IBAT	*Slc10a2*	0.1	0.001
ACAT2	*Acat2*	1.6	0.001	**Excretion**			
ACAT1	*Acat1*	1.5	0.020	ABCG5	*Abcg5*	0.6	0.010
Uptake				**Transcription factors**			
VLDLR	*Vldlr*	32.0	<0.001	**Protein**	**Gene**	**Fold change**	**Padj**
LRP	*Lrp11*	2.5	0.002				
LRP	*Lrp1*	0.6	0.010	HNF4A	*Hnf4a*	1.6	0.001
SORT-1	*Sort1*	0.5	0.004	PGC1alpha	*Ppargc1a*	0.6	0.007
LRP	*Lrp2*	0.2	0.002	CAR	*Nr1i3*	0.6	0.009

Mice received a Western type diet without or with 30 000 ng/g/d PFOA and livers were collected after a 4-hour fast after the VLDL-clearance experiment (experiment 2) at t=4 weeks. Total RNA was extracted from liver of individual mice (n=8 mice per group) and gene expression analysis was performed using the Illumina Nextseq 500. A selection of genes involved in lipid metabolism is depicted with only those DEGs with an adjusted P-value <0.05. Data represent fold change as compared with the control. Abbreviations: Padj, adjusted P-value; TG, triglycerides; FA, fatty acids; VLDL, very-low-density-lipoprotein; HDL, high-density-lipoprotein.

PFOA regulated pathways related to lipid and xenobiotic metabolism, coagulation, and inflammation

To further investigate the mechanism by which PFOA affects lipid metabolism and to explore its effect on other biological processes, pathway analysis was performed in the liver. The total number of DEGs was assessed (**Table 5**) and used to identify overlap between the various treatments and PFOA-specific molecular responses.

There were no statistically significant changes in gene transcripts in the liver with the low PFOA dose group at 10 ng/g/d. In silico prediction of transcription factor activity in the liver (**Table 6**), based on the DEGs (padj <0.05), predicted activation of genes regulated by PPARα (p=1E-75, Z-score 6.5) at 30 000 ng/g/d PFOA dose. This dose of PFOA also activated the transcription factor PXR (Nr1i2), a nuclear receptor that functions as sensor of endobiotic and xenobiotic substances. Analysis of the magnitude of the effect of PFOA on these 2 master regulators of PFOA-induced gene expression showed that these upstream regulators are activated in the order PPARα ≥ PXR.

In addition to modulation of lipid and xenobiotic metabolism, the 30 000 ng/g/d PFOA dose displayed significantly regulated pathways related to coagulation and inflammation (**Table 7**). With the 300 ng/g/d PFOA dose group, there were also statistically

Table 5 The number of DEGs in the liver

	pval<			padj<		
	0.05	**0.01**	**0.001**	**0.05**	**0.01**	**0.001**
Control vs 10 ng/g/d	293	63	12	2	2	1
Control vs 300 ng/g/d	1109	379	103	20	4	3
Control vs 30 000 ng/g/d	5170	3603	2364	3379	2370	1640

Mice received a Western-type diet without or with 10, 300 or 30 000 ng/g/d PFOA, mRNA was isolated from liver tissue, and after further processing next generation sequencing analysis was performed. The table indicates the number of DEGs when compared to control. DEGs depicted in green were used for the pathway analysis in IPA (n=8 mice per group). Padj <0.05 indicates a higher level of stringency as compared to Pval <0.01. Abbreviations: DEGs, differentially expressed genes; pval, p-value; padj, adjusted p-value; IPA, ingenuity pathway analysis.

Table 6 In silico prediction of transcription factor activity based on the expression changes of known target genes at 30 000 ng/g/d PFOA dose

	Upstream regulator	Activation state	*Z*-score	P of overlap
PPAR	Peroxisome proliferator-activated receptor α (PPARα)	activated	6.5	1E-75
HNF1A	Hepatocyte nuclear factor 1 homeobox A	inhibited	-3.6	1E-26
HNF4A	Hepatocyte nuclear factor 4 alpha	inhibited	-2.4	1E-25
ESR1	Estrogen receptor 1	activated	2.4	1E-25
NFE2L2	Nuclear factor, erythroid 2 like 2	activated	3.3	1E-22
NR1L2	Pregnane X receptor (PXR)	activated	5.4	1E-20

Mice received a Western type diet without or with 30 000 ng/g/d PFOA, mRNA was isolated from liver tissue and gene expression analysis was performed using the Illumina Nextseq 500. To determine the activation status of transcription factors, an upstream regulator analysis was performed. A positive Z-score >2 indicates activation and a negative Z-score <-2 inhibition. All DEGs with an adjusted P-value <0.05 were used for the analysis (n=8 mice per group).

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significant changes in the gene transcripts related to hepatic activation of inflammation and immune responses (**Table 7**). Because the biological processes of inflammation and immune response are complex (52) and involve various organs other than liver, the biological significance of these gene transcripts, as they relate to the assessment of cardiovascular risk, remains unclear.

Table 7 Hepatic pathways significantly regulated at 30 000 ng/g/d PFOA dose

30 000 ng/g/d PFOA		300 ng/g/d PFOA	
Canonical pathway	**P-value**	**Canonical pathway**	**P-value**
FXR/RXR activation	1E-15	Phagosome formation	1E-7
LPS/IL-1 mediated inhibition of RXR function	1E-13	Leukocyte extravasation signaling	1E-6
Stearate biosynthesis I (animals)	1E-11	Role of pattern recognition receptors in recognition	1E-6
Fatty acid β-oxidation I	1E-10	Role of NFAT in regulation of the immune response	1E-6
Coagulation system	1E-10	Production of nitric oxide and reactive oxygen species	1E-6
LXR/RXR activation	1E-9	FCγ receptor-mediated phagocytosis in macrophages	1E-5
Acute Phase Response Signaling	1E-8	Dendritic cell maturation	1E-5
Tryptophan degradation III (Eukaryotic)	1E-7	Natural killer cell signaling	1E-5
Superpathway of citrulline metabolism	1E-7	Virus entry via endocytic pathways	1E-5
Complement system	1E-7	CD28 signaling in T helper cells	1E-5
Intrinsic prothrombin activation pathway	1E-6	IL-8 signaling	1E-5
Estrogen biosynthesis	1E-6	TREM1 signaling	1E-4
Bile acid biosynthesis, neutral pathway	1E-6	CTLA4 signaling in cytotoxic T lymphocytes	1E-4
Role of tissue factor in cancer	1E-6	Macropinocytosis signaling	1E-4
Isoleucine degradation I	1E-6	T cell receptor signaling	1E-4
Glutathione-mediated detoxification	1E-6	NF-κB activation by viruses	1E-4
Atherosclerosis signaling	1E-6	Tec kinase signaling	1E-4
Glutaryl-CoA degradation	1E-5	Reelin signaling in neurons	1E-4
Triacylglycerol biosynthesis	1E-5	Granulocyte adhesion and diapedesis	1E-4
Aryl hydrocarbon receptor signaling	1E-5	fMLP signaling in neutrophils	1E-4
Citrulline biosynthesis	1E-5	Epoxysqualene biosynthesis	1E-4
Nicotine degradation III	1E-5	Endothelin-1 signaling	1E-4
Xenobiotic metabolism signaling	1E-5	CD40 signaling	1E-4
Superpathway of melatonin degradation	1E-5	Inflammasome pathway	1E-3
PXR/RXR activation	1E-5	PKCL signaling in T lymphocytes	1E-3

Mice received a Western type diet without or with 30 000 ng/g/d PFOA or 300 ng/g/d, mRNA was isolated from liver tissue and gene expression analysis was performed. Differentially expressed genes (DEGs) (**Table 5**) were used as input for pathway analysis through ingenuity pathway analysis (IPA) suite. All DEGs with an adjusted P-value <0.05 were used for the analysis of the 30 000 ng/g/d dose group, and all DEGs with an P-value <0.01 were used for the analysis of the 300 ng/g/d PFOA dose group. The top 25 most relevant canonical pathways are shown (n=8 mice per group). Abbreviations: FXR, farnesoid X receptor; RXR, retinoid X receptor; LPS, lipopolysaccharides; LXR, liver X receptor; PXR, pregnane X receptor; DEGs, differentially expressed genes.

Discussion

In this study, we have investigated the effects of 3 different doses of PFOA on plasma lipid levels and lipid metabolism in APOE*3-Leiden.CETP mice, a mouse model with a human-like lipoprotein metabolism (31). At the end of the study, the low dose group at 10 ng/g/d resulted in a mean plasma PFOA concentration of approximately 50 ng/mL, which is similar to the range reported in a community population in the mid-Ohio river valley area that had known PFOA exposure in its drinking water (median 27 ng/mL, mean 80 ng/mL) (8). Likewise, the mid-dose group at 300 ng/g/d resulted in a plasma concentration of approximately 1500 ng/mL which is within the range reported in different occupational studies among fluorochemical production workers (9,10,12,15). At 30 000 ng/g/d PFOA achieved a plasma concentration that approached the level reported in the phase 1 clinical trial where a decrease in total cholesterol was clearly observed to occur (30).

This differentiation of plasma PFOA concentrations is a major strength of this study to understand lipid-related associations, or lack of, in human observational and experimental studies. Plasma PFOA concentrations were translatable to the human exposure scenarios and we conclude that, using the APOE*3-Leiden.CETP mouse model, plasma PFOA levels at either 50 ng/mL (community drinking water exposed) or 1500 ng/mL (occupationally exposed) did not alter plasma lipids. PFOA exposure did decrease plasma TG, TC, and non-HDL-C levels and increased HDL-C level when plasma PFOA concentrations reached 90 663 ng/mL (at the end of the 4-week treatment) or 144 000 ng/mL (at the end of the 6-week treatment). We have demonstrated that environmentally and occupationally relevant PFOA exposures did not affect plasma lipids or lipoprotein metabolism using this mouse model. Our data are consistent with the findings by Convertino et al. (30) that high serum or plasma PFOA levels resulted in lower cholesterol levels. Our current study data do not show an increase in cholesterol at environmental or occupational levels of PFOA exposure as shown in some observational epidemiological studies, suggesting these findings are likely associative rather than causal.

Consistent with our data, toxicological PFOA concentrations (>30 000 ng/g/d or 0.02% wt/wt) in mice and rats decreased plasma TC (20,22,23). While the major lipid in wild-type rodents are limited to HDL-C, it is interesting that cholesterol contained in the HDL-C fraction was reported to be increased in C57BL/6 and BALB/c mice fed a high cholesterol and high fat diet containing PFOA (560 µg/kg/d) (21). However, given the inherent difference in rodent lipid metabolism (vs human), it is difficult to extrapolate these results to the human situation, emphasizing the importance to select an animal model resembling human lipid metabolism (31). The APOE*3-Leiden.CETP mouse used in our study is a well-characterized model for its human-like lipoprotein metabolism with delayed apoE-LDLR clearance and expression of CETP, and these characteristics are absent in wild-type rodents, including C57BL/6 and BALB/c mice.

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Unlike wild-type rodents, nonhuman primates and humans express CETP. Butenhoff et al., (24) found no effect on lipid levels when cynomolgus monkeys were administered with daily oral doses of PFOA for 6 months (24). In that study, serum PFOA concentration approximated $158\,000 \pm 100\,000$ ng/mL; however, there was no distinction made between cholesterol in non-HDL-C or HDL-C hence precluding direct comparison with the present data. In humans, Convertino et al. (30) reported a decline in TC and LDL-C with high (toxicological exposure) plasma concentrations of PFOA, however, unlike our study, they did not observe any change in HDL-C. This discrepancy could be due to the higher CETP activity measured in APOE*3-Leiden.CETP mouse plasma than human (**Figure 3B**). The concomitant increase in HDL-C observed in our study resulted from downregulation of CE transfer activity in plasma by PPARα activation (of which PFOA has been shown to be an activator), the strong downregulation of *Scarb1* (the principle gene in HDL-C clearance) and *Lipc* (plays a role in remodeling of HDL), and by the increased expression of *Pltp* (resulting in formation of larger HDL particles) (39,53,54).

Most reports studying the effects of PFOA in experimental animals do not provide a mechanistic explanation for the observed changes in lipoprotein metabolism. Our studies revealed that high PFOA exposure decreased (V)LDL levels by severely impairing the production of VLDL and increasing VLDL clearance by the liver through increased LPL-mediated lipolytic activity, accompanied by hepatomegaly with cellular hypertrophy. Gene expression and pathway analysis confirmed that lipid metabolism was regulated by PFOA mainly through activation of PPARα. Although not evaluated in this study, the hepatocellular hypertrophy is likely reversible, as has been reported in rats (50) and monkeys (24).

The increased VLDL clearance accompanied by augmented uptake of FFAs by the liver and LPL-expressing organs, such as sWAT and BAT, can be explained by upregulation of *Lpl* and downregulation of an inhibitor of LPL (*Apoc3*), resulting in increased plasma LPL activity. The strongly decreased VLDL-TG and VLDL-apoB production rate (equally diminished by as much as 85%) is not caused by PPARα activation as fenofibrate enhances VLDL-TG secretion (28) or by reduced availability of lipids for VLDL synthesis because hepatic lipid content was not reduced by PFOA. More likely, PFOA prevents VLDL particle formation and secretion from the liver by reduced apoB *de novo* synthesis caused by decreased *Apob* mRNA expression. In line with this contention, treatment of cultured rat hepatocytes with PFOA decreased the VLDL secretion through disturbance of the association of apoB48 with VLDL particles, a process independent of PPARα (55), and toxicological PFOA exposure reduced apob100 mRNA expression in BALB/c mice (56) as well. Therefore, we conclude that at toxicological PFOA exposure the decreased plasma TC and TG levels result from increased VLDL clearance and diminished VLDL-TG and VLDL-apoB production, the latter caused by a reduced supply of apoB substrate which is essential for the assemblage of the VLDL particles.

Fecal neutral sterol and bile acid excretion, the latter as marker of hepatic bile acid synthesis, were not affected by PFOA despite a decrease in Cyp7a1 mRNA expression,

indicating that there is sufficient supply of substrate for bile acid synthesis. Consistently, we found no changes in hepatic lipid content, implying that despite reduced VLDL production, hepatic cholesterol and TG homeostasis is maintained. In contrast, the mass of adipose tissue was decreased in spite of enhanced plasma lipolytic activity. LPL-mediated delivery of VLDL-TG–derived FA is a strong determinant of WAT mass and obesity (57). However, the reduced VLDL-TG production limits the availability of substrate for LPL on peripheral tissues, leading to less FA delivery to WAT and skeletal muscle. This can explain the reduced pWAT mass induced by toxicological PFOA exposure, accompanied by a reduction of plasma FFAs and glycerol that are mainly derived from TG lipolysis in adipose tissue.

Gene expression and pathway analysis revealed that FA oxidation and individual genes involved therein, all under control of PPARα, were enhanced, in line with previous reports in mice (58), rat (50) and human hepatocytes (25,59). In silico prediction of transcription factor activity predicted PPARα as the principal transcription factor regulated by toxicological PFOA not only based on the regulation of lipid metabolism-related genes, but of all DEGs in the liver. Upstream regulator analysis also predicted the involvement of the xenosensor receptor PXR (*Nr1I2*) in the regulation of biological processes, whereas CAR (*Nr1I3*) mediated processes were affected to a much lesser extent, in line with literature (26). Next to its role in xenobiotic metabolism, PXR activation is also involved in lipid metabolism, as it reduces the hepatic expression of *Apoa1*, *Lcat*, and *Hl*, players in the formation, maturation, and remodeling of HDL-C in APOE*3-Leiden.CETP mice (38) as also observed in the present study. While accumulation of TG and cholesterol in the liver can be induced by constitutive PXR expression (60) or the PXR agonist PCN (38), it was not observed in our study, most likely because the PXR-induced effect was negated by the strong PPARα activation, leading to decreased hepatic lipid content in APOE*3-Leiden. CETP mice (28), provoked at the applied PFOA exposure at high (toxicological) level.

At 30 000 ng/g/d PFOA, inflammation-related gene pathways were observed to have statistically significant changes. Activation of both PPARα and PXR is known to attenuate the inflammatory response (61–63), and toxicological exposure to PFOA has been reported to reduce inflammatory pathways and responses (64–67). In contrast, PFOA at 300 ng/g/d upregulated pathways involved in inflammatory and immune response processes. The physiological consequences hereof, however, are unknown and require further research.

In conclusion, we have demonstrated that in APOE*3-Leiden.CETP mice, dietary PFOA exposure at 30 000 ng/g/d reduced plasma TG and TC levels by affecting VLDL-TG production through decreased apoB synthesis and by increasing VLDL clearance. This was not observed with lower PFOA doses. Our data confirmed the findings from a phase 1 clinical trial in humans that demonstrated high serum or plasma PFOA levels resulted in lower cholesterol levels. The study findings do not show an increase in cholesterol at environmental or occupational levels of PFOA exposure, thereby indicating these findings are associative rather than causal.

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Disclosures

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