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ORIGINAL ARTICLE

Exposure to perfluorononanoic acid combined with a low-dose mixture of 14 human-relevant compounds disturbs energy/lipid homeostasis in rats

Kasper Skov[1] · Kristine Kongsbak[1] · Niels Hadrup[1] · Henrik Lauritz Frandsen[1] ·
Terje Svingen[1] · Jørn Smedsgaard[1] · Karine Audouze[2,3] · Aron Charles Eklund[2] ·
Anne Marie Vinggaard[1]

Abstract Humans are constantly exposed to a significant number of compounds and many are readily detected in human body fluids. Worryingly, several of these compounds are either suspected to be, or have already been shown to be harmful to humans either individually or in combination. However, the potential consequences of low-dose exposure to complex mixtures remain poorly understood. We have profiled the effects on rat blood plasma and liver homeostasis using metabolomics and transcriptomics following 2-week exposure to either a mixture of 14 common chemicals (Mix), perfluorononanoic acid (PFNA) at low (0.0125 mg/kg/day) or mid (0.25 mg/kg/day) doses, or a combination of Mix and PFNA. In blood plasma, 63 and 64 metabolites were significantly changed upon exposure to Mix alone or PFNA + Mix, respectively. Twelve of the metabolites were identified and comprised mainly lipids, with various lipid classes differentially affected across study groups. In the liver, expression of 182 and 203 genes—mainly related to energy homeostasis and lipid metabolism—were differentially expressed upon exposure to PFNA alone or PFNA + Mix, respectively. In general, Mix alone affected lipid metabolism evident in blood plasma, whereas effects on lipid metabolism in the liver were mainly driven by PFNA. This study verifies that a chemical mixture given at high-end human exposure levels can affect lipid homeostasis and that the combined use of metabolomics and transcriptomics can provide complimentary information allowing for a detailed analysis of affected signaling pathways.

Keywords Perfluorononanoic acid · Metabolomics · Transcriptomics · Lipid homeostasis · Mixture toxicology

Kasper Skov and Kristine Kongsbak contributed equally to the work.

✉ Anne Marie Vinggaard
annv@food.dtu.dk

[1] National Food Institute, Technical University of Denmark, Mørkhøj Bygade 19, 2860 Søborg, Denmark

[2] Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, 2800 Kongens Lyngby, Denmark

[3] Inserm UMR-S 973, Molécules Thérapeutiques In Silico, Paris Diderot University, Paris, France

1 Introduction

Humans, particularly those living in industrialized countries, are continuously exposed to a plethora of compounds through foods, cosmetics, pharmaceuticals, air inhalation and more (Monosson 2005). A large number of studies have reported on the presence of multiple compounds in human body fluids (Calafat et al. 2007; NHANES 2013), clearly showing that they are taken up by the body through various routes. Also, epidemiological studies have shown strong associations between compound mixtures and diseases, for instance in relation to human reproduction (Krysiak-Baltyn et al. 2012; Taylor et al. 2014). Therefore, since animal studies have shown effects of human relevant mixtures of environmental compounds given at doses close to No Observed Adverse Effect Levels (NOAELs) for single compounds (Christiansen et al. 2008, 2012; Axelstad et al. 2014), the presence of many of these chemicals simultaneously is of real concern to human health.

The traditional approach for toxicological testing aims at understanding the effect(s) of a single compound on biological systems, from cells (in vitro) to animals

(in vivo). The compound is typically investigated at varying doses in order to obtain information on parameters such as the NOAEL. A few studies on rats exposed to chemicals or chemical mixtures at doses representing human exposure levels have been reported (Moser et al. 2006; Chen et al. 2014; Hadrup et al. 2015). These studies suggest that even at low doses, certain compounds can have effects on biological systems, for instance the plasma metabolome. However, there still is a significant gap in knowledge regarding effects of low-dose chemical mixtures on either the metabolome or transcriptome at the 'omics' level.

'Omics' technologies have provided new approaches to evaluate the effects of toxic compounds. For instance, fluorinated compounds have been shown to change the lipid metabolism of both rats and zebrafish (Fang et al. 2012a; Zhang et al. 2012) and endocrine disrupting compounds such as bisphenol A have been shown to cause changes to the metabolome even at doses far below the NOAEL (Chen et al. 2014). In general, changes in the transcriptome and metabolome are detectable even after exposure to a low dose of certain compounds, which suggests that these methods can be valuable tools for understanding how the compounds affect the organism where obvious phenotypes are absent (Chen and Kim 2013). Furthermore, it has been shown that a mixture of compounds at low doses can result in a marked effect even when individual compounds show no detectable effect and that this effect is marked even if they 'only' act additively (Silva et al. 2002).

We wanted to investigate the effects of a mixture of compounds (Mix) alone and together with increasing doses of the perfluorocarboxylic acid, perfluorononanoic acid (PFNA), as well as the effects of PFNA alone. The Mix was composed of 12 environmentally relevant endocrine disrupting compounds at high-end human exposure levels (Christiansen et al. 2012) and two food ingredients in doses corresponding to a high-end daily intake of grapefruit and licorice. We hypothesized that adverse effects would be observed with Mix alone or when combined with low dose PFNA. By use of two complementary omics methods, metabolomics and transcriptomics, we profiled the effects in blood plasma and livers of exposed rats and compared them to adverse effects observed at higher PFNA doses. We found significant changes both to the metabolome, as detected in plasma, and the liver transcriptome following exposure to Mix and PFNA.

2 Materials and methods

2.1 Compounds and dosing

Compounds were selected as previously described (Hadrup et al. 2015). Animals were dosed with a mixture of 12 compounds: bisphenol A, butylparaben, dibutyl phthalate (DBP), bis(2-ethylhexyl)phthalate (DEHP), 4-methylbenzylidene camphor, octyl methoxycinnamate, dichlorodiphenyldichloroethylene (p,p'-DDE), epoxiconazole, linuron, prochloraz, procymidone, vinclozolin) and described elsewhere (Christiansen et al. 2012; Hadrup et al. 2013), along with the two food components; glabridin from licorice and bergamottin from grapefruit (Hadrup et al. 2015), both known to inhibit the activity of cytochrome P450s (CYPs) metabolizing hormones and chemicals. The total dose of the Mix was 2.5 mg/kg/day and the ratio of the compounds is presented in Supplementary Table 1. The dose of Mix was based on previously observed endocrine disruption (Christiansen et al. 2012), albeit 24-times lower herein to reflect human exposure corrected for different body surface areas of rat and human. In addition to Mix, animals were exposed to three different doses of PFNA, the lowest dose corresponding to a high-end human exposure level (Lau et al. 2007). The two lowest doses were Low = 0.0125 mg/kg/day and Mid = 0.25 mg/kg/day. A high dose of PFNA (5 mg/kg/day) was part of the study as well but was excluded from the mechanistic analyses in this paper due to the observed severe toxicity (Hadrup et al. 2015) that would hamper the interpretation of data.

2.2 Animals

The animal study has been described previously (Hadrup et al. 2015). In brief, male Wistar Hannover Galas rats at 6 weeks of age were housed two per cage with a 12-h light/dark cycle and ad libitum access to acidified tap water and standard diet. The animals received vehicle (corn oil) or test substances once daily by gavage for 14 days. Fifty-four rats were randomly assigned into six groups (Table 1). The last dose was administered to each animal 75–105 min before euthanization. The rats were anaesthetized in CO_2/O_2 prior to decapitation. Plasma was isolated from heparinized neck blood by centrifugation at $1000g$ at 4 °C for 10 min and subsequently stored at −80 °C. Livers were weighed and snap-frozen in liquid nitrogen.

2.3 Metabolomics

The procedure was as previously described (Skov et al. 2014). In brief, phospholipids were adsorbed on a

Table 1 Acronyms used for the different treatment groups and respective number of animals per group

	Without Mix	With Mix	Acronym
Control	10	8	Control/mix
0.0125 mg/kg/day	10	10	Low PFNA ± mix
0.25 mg/kg/day	8	8	Mid PFNA ± mix

phospholipid SPE column (Supelco, Sigma-Aldrich). The eluate was collected, dried and extracted using first 200 µl heptane to isolate the lipids followed by 200 µl methanol to extract the more polar compounds. The phospholipids were eluted from the SPE column using 300 µl 10 % NH_4OH in methanol. The phospholipid, lipid and the polar fractions were analyzed by an HPLC system combined with a Maxis Quadrupol Time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany).

Data were analyzed with Profile Analysis 2.1 (Bruker Daltonics, Bremen, Germany). Data were extracted using the "find molecular features" algorithm in a mass range from 50 to 1100 m/z value. The noise was reduced by removing peaks that were present in <50 % of the samples among all treatment groups and at the same time had a peak intensity of ≤ 3000. The calculations were carried out in R (R Core Team 2012). The data were uploaded to MetaboAnalyst.ca (Xia et al. 2012) and analyzed with t test, principal component analysis, and partial least squares discriminant analysis (PLS-DA). The accurate masses of significantly different metabolites were searched for in databases such as the human metabolome database (HMDB) (Wishart et al. 2009). The identities of the compounds were verified by comparison of MS/MS patterns with data from the databases HMDB, METLIN (www.metlin.scripps.edu) (Smith et al. 2005), LIPID MAPS (www.lipidmaps.org) (Sud et al. 2007) and MassBank (www.massbank.jp) (Horai et al. 2010).

2.4 Statistical analysis

Initial analyses of the metabolome data were performed on the Metaboanalyst server (Xia et al. 2012). Here, a one-way analysis of variance (ANOVA) comparing PFNA-treated animals to control animals and PFNA + Mix-treated animals to control, respectively, formed the basis for initial selection of significantly altered metabolites. All p-values were adjusted using false discovery rate (FDR) according to the protocol implemented in the Metaboanalyst workflow, and 0.05 was used as cut-off for statistical significance. The statistically significantly altered metabolites were subsequently analyzed and plotted using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). The D'Agostino and Pearson omnibus normality test was used to test for normality of the data. If data were normally distributed, an ANOVA was performed using Dunnett's multiple comparisons test to adjust the p-values. If data were not normally distributed, data were log-transformed and, if normally distributed after transformation, analyzed by ANOVA. If not normally distributed, a non-parametric Kruskal–Wallis test followed by a Dunn's multiple comparisons test was conducted. The criteria for statistical significance was $p < 0.05$, $p < 0.01$ and

$p < 0.001$ denoted *, ** and ***, respectively. Statistical comparisons not applicable to ANOVA tests were carried out using an unpaired, two-tailed Student's t test.

2.5 Transcriptomics

Total RNA from six rat livers each from vehicle control, Low PFNA + Mix, and Mid PFNA ± Mix groups were separately converted into labeled cRNA and applied to the One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) version 6.5 (Agilent Technologies, Santa Clara, CA). Labeled cRNA from each rat was hybridized to Agilent Whole Rat Genome Oligo Microarrays (G4122F) for 17 h at 65 °C. The hybridized microarrays were scanned using an Agilent DNA Microarray Scanner and evaluated using the Feature Extraction software version 10.7.3.1 according to protocol GE1_107_Sep09 (Agilent Technologies) to generate feature extraction files for further analysis. Reads were quality controlled by the software prior to release of the data. Arrays that did not pass quality control were removed from the dataset. Based on the quality control reports, two of the six microarrays from the Mid PFNA + Mix group were excluded from further analysis. The remaining arrays, six from each of control, Low PFNA + Mix, and Mid PFNA and the remaining four from Mid PFNA + Mix were found to be of high quality.

Extracted data were analyzed using the limma software package (Smyth 2004, 2005) in R (R Core Team 2012). Data were background corrected using the 'normexp' method (Ritchie et al. 2007) and normalized between arrays using quantile normalization (Smyth and Speed 2003) prior to statistical analyses. Within-array replicate probes were replaced with the average expression level. To identify treatment-specific gene effects, we fitted a linear model for each gene and applied empirical Bayes statistics (Smyth 2004) for each relevant two-group comparison. The FDR was controlled using the Benjamini–Hochberg method (Benjamini and Hochberg 1995). Reported p-values for the significantly differentially expressed genes from the transcriptomics analysis were all adjusted, and p-values ≤ 0.05 were considered statistically significant.

2.6 Pathway analysis

Transcription data were analyzed with QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity). IPA was used to map significantly differentially expressed gene probes to genes, and expression values were used for prediction of the involvement of the differentially expressed genes in functional networks, pathways, and diseases, and for graphical representations. Using the Fisher's Exact Test, we analyzed

the overlap between the differentially expressed genes in our dataset and genes known to be involved in disease networks, pathways, and diseases available in the Ingenuity Knowledgebase. *p*-values ≤ 0.05 were considered statistically significant.

3 Results and discussion

3.1 Rats exposed to low/medium levels of a 14-chemical Mix, PFNA, or both displayed no macroscopic phenotypes

Our main objective was to determine if exposure to low doses of PFNA with or without a background exposure to a low-dose chemical mixture (Mix) can have undesirable effects on the metabolome and transcriptome of animals otherwise displaying little to no phenotypic abnormalities. For this, we chose to analyze blood and liver samples from male rats that had been exposed to various levels of chemicals for a period of 14 days during juvenile age. This in vivo experiment has been described previously with a focus on low dose hormonal changes and pathological findings at high dose level (Hadrup et al. 2015). In the initial assessment of the animals, exposure to high levels of PFNA, with or without Mix, indicated hepatic steatosis of the liver; a state of retention of lipids in the liver. Animals exposed to lower levels of PFNA ± Mix, which encompasses the dose-groups included in this study, showed no obvious morphological phenotypes. However, significant plasma levels of PFNA after the exposure period were measured to be 1.1 and 30 µg/ml for Low PFNA + Mix and Mid PFNA + Mix, respectively. In animals exposed to Low-, and Mid PFNA only, plasma concentrations were 0.4 and 40 µg/ml, respectively. The former is in the range of, or up to six times the human high-end combined exposure to PFOA, PFNA and PFOS (67.6–824 ng/ml) (Emmett et al. 2006; Lau et al. 2007; NHANES 2013; Hadrup et al. 2015). Thus, the addition of Mix significantly increased the plasma levels of PFNA (2.8-fold for Low PFNA), indicating altered ADME (absorption, distribution, metabolism, excretion) properties of PFNA when Mix is co-administered.

The dose of Mix was calculated from a high-end exposure level in the European population (Christiansen et al. 2012) and corrected for body surface area of rats compared to humans (Hadrup et al. 2015). Surprisingly, none of the compounds in Mix, or their corresponding metabolites, were found when extracted ion chromatograms were created based on their accurate mass. This could be due to rapid metabolism of the compounds or that the levels were below the limit of detection in the LC–MS analysis. Nevertheless, as clear effects of PFNA levels were observed when co-administered with Mix, we chose to include all

the different combinations of low- to mid-range exposure groups in our analyses aimed at discerning more subtle molecular phenotypes.

3.2 The blood plasma metabolome of rats exposed to low/medium levels of chemical mixtures, including PFNA, was significantly altered

In order to establish any potential effects caused by PFNA exposure with or without a Mix background, any effects caused by Mix alone was analyzed first. When compared to control animals, Mix exposure significantly affected 63 out of a total of 882 molecular features. As shown in Fig. 1, affected phospholipids were lysophosphatidylcholines such as (lyso-PC)(20:4) and lyso-PC(18:2), whereas affected neutral lipids included diacylglycerols (DG) such as DG(18:1/18:3) and DG(18:2/16:0), all depressed in serum of exposed animals. Plots of all significantly altered metabolites are shown in Supplementary Figs. 1 and 2, again with most of the affected metabolites being depressed relative to control.

Altered lipid metabolism is a common response to xenobiotic exposure (Karami-Mohajeri and Abdollahi 2011; Zhang et al. 2013; Androutsopoulos et al. 2013; Zhang et al. 2014). An initial stress-response to a xenobiotic insult can increase carbohydrate metabolism to meet a changing energy requirement, subsequently met by increased lipid and protein metabolism (Karami-Mohajeri and Abdollahi 2011). The general decrease in plasma lipid levels observed in the Mix-exposed rats could reflect this situation, particularly since the exposure lasted for a prolonged period (14 days), potentially resulting in effects such as hepatic injury. Interestingly, two of our identified lysophosphatidylcholines, lyso-PC(18:2) and lyso-PC(20:4) have both been identified as potential blood biomarkers for drug-induced hepatic phospholipidosis (Saito et al. 2014), and in both instances the response is reduced levels. Then, as drug-induced phospholipidosis has been associated with liver inflammation and fibrosis (Rigas et al. 1986; Lewis et al. 1989), it could suggest that we are observing compromised livers in our rats exposed to Mix, albeit not observed at the macroscopic level.

In animals exposed to Low PFNA only, we also observed changes to the blood metabolome, albeit different to what was observed with Mix only. Using PLS-DA of all the three fractions, we were able to separate the various exposure groups, with the lipid fraction depicted in Fig. 2. There was a clear difference between Low PFNA and Mix groups, but it was not possible to positively identify the specific metabolites responsible for this separation.

In all 30 metabolites separated Mix-exposed animals from animals exposed to Low PFNA (Supplementary Fig. 1). Mix alone altered 22 of these metabolites. For 6 out of 22 metabolites the concomitant exposure to Low PFNA
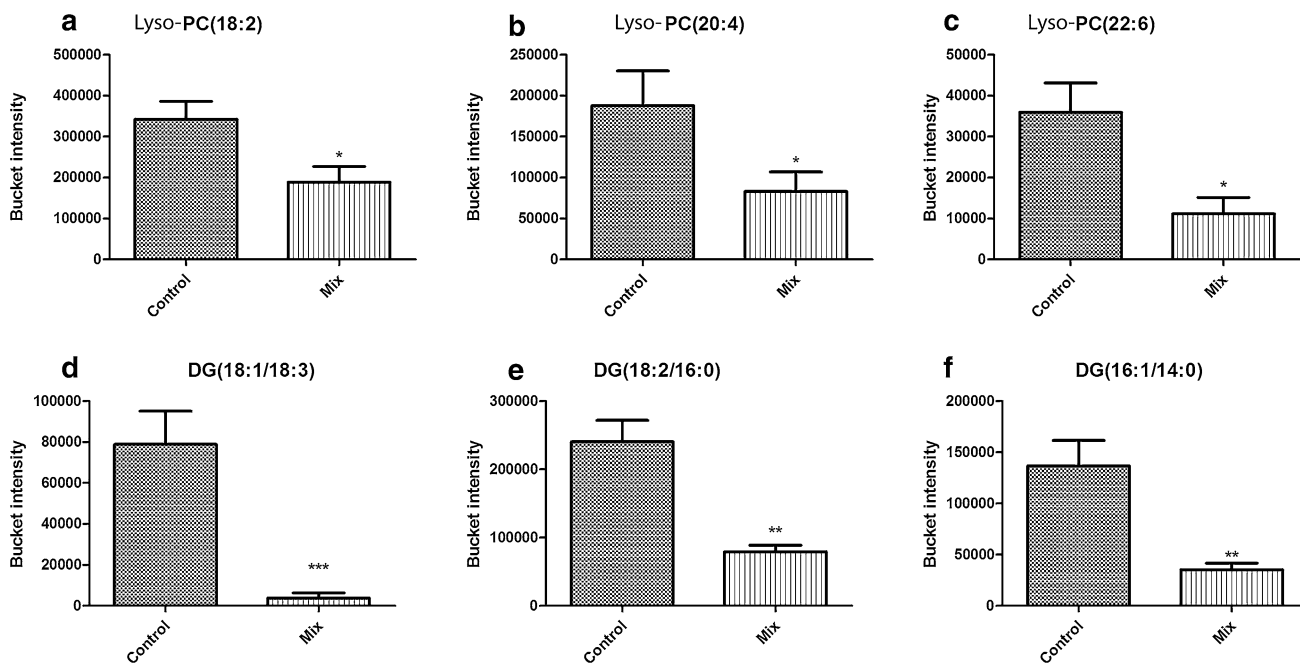
Fig. 1 The six most significantly different metabolites in the Mix group compared to the control group. In total, the levels of 63 metabolites were found to be significantly different between the two groups (t test). The depicted metabolites belong to two metabolite classes, diacylglycerols (DG) and lyso-phosphatidylcholines (lyso-PC)

Fig. 2 PLS-DA plot of the heptane fraction correlating the four groups Control ($n = 10$), Low PFNA ($n = 10$), Low PFNA + Mix ($n = 10$) and Mix ($n = 8$). The plot illustrates that Mix accounts for a major part of the variance in the dataset
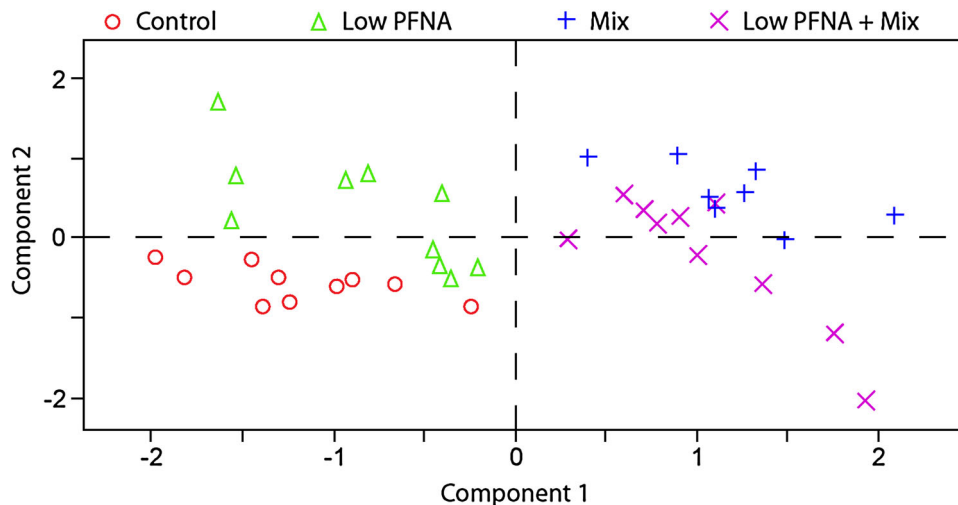
enhanced the effect on the metabolites. In the animals simultaneously exposed to Low PFNA and Mix, we observed a general depression of lipid concentrations in blood plasma, primarily diacylglycerols and the free fatty acid lignoceric acid (Fig. 3a, b). The only metabolite directly affected by Low PFNA, but not by Mix was the steroid hormone corticosterone, which was significantly elevated compared to control animals (Fig. 3c). PFNA-exposure has previously been shown to elevate plasma levels of both adrenocorticotropic hormone and cortisol in mice, though only at the highest dose of 5 mg/kg/day (Fang et al. 2008). Although it

is not clear why adrenocorticotropic hormone, and subsequently glucocorticoids, are elevated by PFNA-exposure, it could be an inflammatory response, as PFNA can induce immunotoxic responses (Fang et al. 2008), and elevated endogenous glucocorticoid levels can subsequently suppress the innate immune response (Liberman et al. 2007). None of the metabolites that were altered by Mix and not by Low PFNA + Mix were identified.

We next performed the same analyses on animals having been exposed to Mid PFNA doses with or without Mix. Within these groups, we identified 24 metabolites with
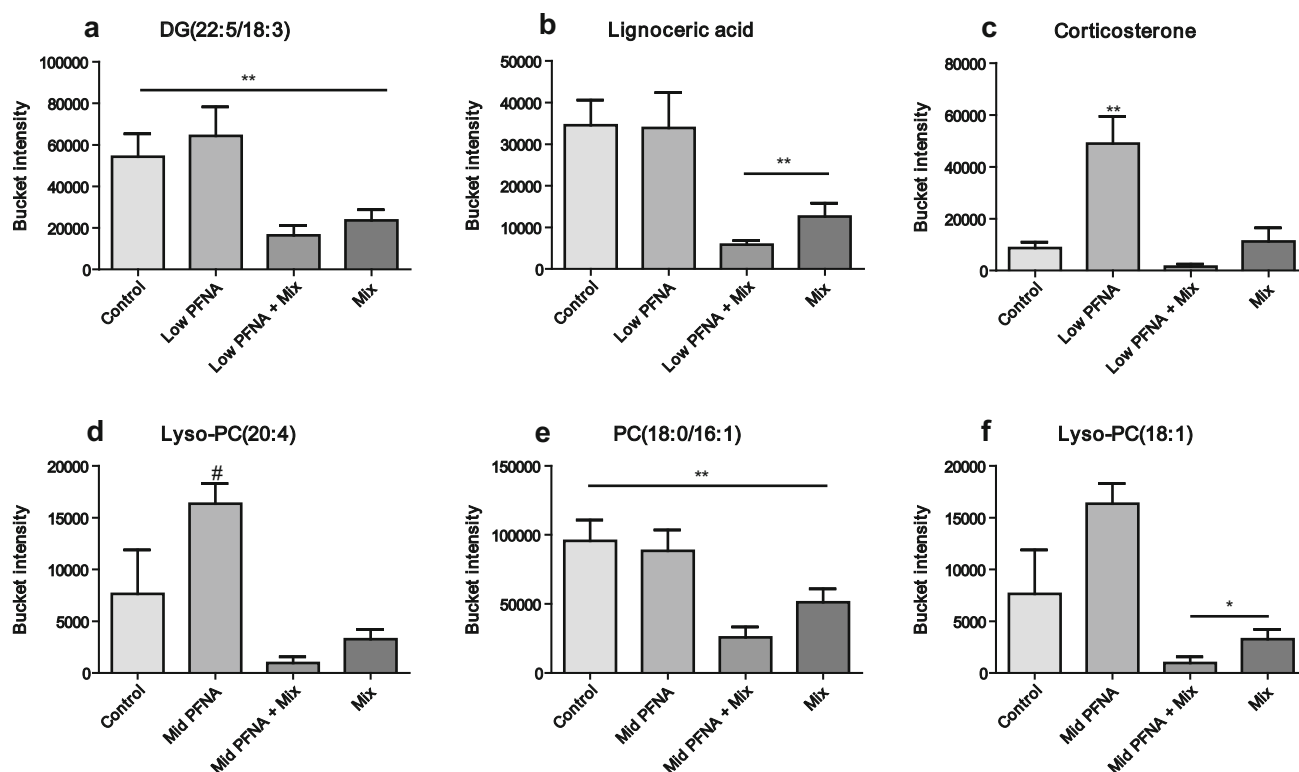
Fig. 3 Representative metabolite changes in the Low PFNA + Mix group. The *plotted* metabolites belong to two metabolite classes; PC (phosphatidylcholine) and DG (diacylglycerol). Metabolite levels were analyzed statistically for each group by comparison to control (*t* test). For corticosterone (**c**) and lyso-PC(20:4) (**d**), PFNA alone caused an increased metabolite level, whereas the combination of Mix and PFNA generally caused a decreased metabolite level (DG(22:5/18:3) (**a**) and PC(18:0/16:1) (**e**)). For lignoceric acid (**b**) and lyso-PC(18:1) (**f**) the effect by Mix + PFNA is stronger than the effect of Mix or PFNA alone. [#]*p*-value of 0.053

significantly changed plasma concentrations, out of which 17 were altered by Mix alone (Supplementary Fig. 2). Three representative metabolites that were significantly changed between groups are shown in Fig. 3d–f. As shown, there was an increase in the plasma level of lyso-PC(20:4) in Mid PFNA exposed animals, but not in those exposed by Mix alone. Exposure to Mix depressed plasma levels of the diacylated phospholipids PC(18:0/16:1), which was further depressed by the simultaneous exposure to PFNA despite Mid PFNA exposure alone not affecting levels of these metabolites (Fig. 3e).

To further investigate the contribution of Mix towards the PFNA-induced effects, we compared the significant metabolites resulting from statistical analysis of Mix versus control (*t* test) and PFNA + Mix versus control (ANOVA). We observed that 24 metabolites were affected by all treatments, out of which 12 were positively identified (Supplementary Fig. 3). These 12 metabolites comprised neutral short-chained lipids such as monoacylglycerols and phospholipids. Mix alone affected the diacylglycerols, whereas the PFNA + Mix groups affected the diacylated phospholipids. Surprisingly, lyso-PC(20:4) and lyso-PC(18:1) were depressed by Mix exposure, elevated by Mid PFNA exposure, but depressed

beyond Mix alone when simultaneously exposed to Mid PFNA and Mix (Fig. 3d, f). Similarly, although not elevated by Mid PFNA exposure, blood plasma levels of lyso-PC(18:0) and lyso-PC(16:1) appeared to be depressed more when exposed to Mid PFNA + Mix than when exposed to Mix alone (Fig. 3e). It is difficult to discern a putative mechanism behind these observations, but it likely reflects the complex regulatory mechanisms underpinning organismal homeostasis, highlighting the importance of employing systems approaches when analyzing effects caused by mixtures of compounds. However, in some ways these metabolite changes are similar to those associated with metabolic syndrome.

Over the last decades, there has been a worldwide increase in metabolic syndrome (World Health Organization 2000), carrying with it an increased risk of developing diabetes and cardiovascular disease. Some studies have shown decreased phospholipid levels in blood plasma of diabetics (Wang et al. 2005; Liu et al. 2013) and an abnormal ratio between lyso-PC and PC levels, which may indicate a systemic change in relation to diabetes (Altmaier et al. 2008). PCs are involved in the biosynthesis of multiple compounds and a decrease in PC levels can reflect increased biosynthesis of lipids such as triacylglycerols

(Lagace and Ridgway 2013). Thus, the changes we observed in the metabolome in rats following exposure to Mix alone and PFNA + Mix were comparable to the differences observed in patients with metabolic syndrome (Ferrannini et al. 2013). Also, lyso-PCs have been shown to be elevated in metabolic syndrome patients (Chen et al. 2011).We observed elevated lyso-PCs for Mid PFNA exposure, but not for the other exposure groups. This could suggest that Mix, or components therein, protects against some of the effects caused by PFNA. However, a more likely scenario is that Mix exposure causes systemic changes that lead to a metabolic response in order to maintain tissue/serum homeostasis or to a suppressed immune response, which subsequently leads to differing responses to PFNA.

The effects observed on lipid homeostasis following exposure to Mix with or without PFNA may be attributed to the presence of glabridin, a naturally occurring isoflavonoid from licorice that has been shown to inhibit the activity of CYP2C9 in the liver (Kent et al. 2002). Studies have shown that CYP2C9 is involved in the regulation of lipid metabolism in the body (Kent et al. 2002; Kirchheiner et al. 2003). Furthermore, people carrying a polymorphism in CYP2C9 have a tendency towards having lower levels of the lipid carriers LDL and HDL (Kirchheiner et al. 2003). A decrease in LDL and HDL may result in decreased levels of plasma lipids, including cholesterol and cholesterol derivatives, as these are carrying lipids in the blood stream (Kent et al. 2002). It could also be attributed to PFNA inhibiting the activity of CYP2C9 similarly to glabridin (U. S. Environmental Protection Agency 2014).

3.3 The liver transcriptome of rats was affected by exposure to chemical mixtures, including PFNA

For global gene expression analyses, we selected to study the livers of animals exposed to Low PFNA + Mix and Mid PFNA with or without Mix relative to control, as we expected little changes in the Mix alone group. As evident in Supplementary Table 3, we observed no statistically significant ($p \leq 0.05$) changes in transcript levels of any gene in livers from Low PFNA + Mix exposed rats. Further, few genes in these groups displayed significant fold-change differences in transcript abundance, with only three genes displaying more than 1.5-fold difference: *Cyp4a11* 1.6-fold, *Cyp2b6* 2.0-fold and *Acot1* 2.1-fold. Allowing for an adjusted p-value ≤ 0.1 without a fold-change cut-off value, 31 genes could be identified as differentially expressed between the exposed and control rat livers, and are included in Table 2, which lists differentially expressed genes related to energy homeostasis. The remaining genes are listed in Supplementary Table 3.

Next we analyzed the transcriptome of livers obtained from rats exposed to Mid PFNA ± Mix. Here, only annotated genes contained in the IPA database were included when compiling lists of differentially expressed genes, thus excluding most expressed sequence tags and genes without adequate literature-based information for robust downstream analysis. At the statistically significant level of $p \leq 0.05$, 203 genes from the PFNA + Mix group and 182 genes from the PFNA only group were annotated as differentially expressed (Supplementary Table 2). Of these, the majority showed an upregulation in the exposed groups as compared to controls.

Based on functional annotation of genes dysregulated in the PFNA ± Mix dosing groups, fatty acid metabolism was the main biochemical function affected (Table 2). For instance, upregulated genes such as *Crot*, *Crat*, *Acox1*, *Ehhadh*, *Hadha*, *Hadhb*, *Decr2*, *Eci2*, *Ech1*, are all involved in peroxisomal fatty acid β-oxidation, whereas *Cpt2*, *Slc25a20*, *Acad11*, *Acadl*, *Acadm*, *Acads*, *Acadvl* are all involved in mitochondrial β-oxidation. Genes associated with lipid transport, fatty acid activation, and peroxisomal transport (*Apoa2*, *Abcd3*, *Cd36*, *Slc27a2*) were also upregulated. Amongst the downregulated genes was Fatty acid binding protein 5 (*Fabp5*). Similar effects on lipid homeostasis have been shown in other studies (Guruge et al. 2006; Rosen et al. 2007; Fang et al. 2012a, b, c). Here, the effects on lipid metabolism seem to be mainly driven by PFNA exposure, as the effects are comparable between animals exposed to Mid PFNA only and Mid PFNA + Mix.

With regards to aerobic respiration, a different transcriptional profile was observed for Mid PFNA + Mix compared to Mid PFNA alone (Table 2). For instance, the citric acid cycle enzymes, aconitase 2 (*Aco2*), isocitrate dehydrogenase 3 (NAD+) beta (*Idh3b*), and succinate-CoA ligase (*Sucla2* and *Suclg1*) were significantly upregulated by Mid PFNA, but not by Mid PFNA + Mix. Only *Aco2* and *Suclg1* were upregulated in both groups relative to control. Similarly, for enzymes in the electron transport chain (*Ndufa10*, *Ndufab1*, *Ndufs3* (complex I), and *Sdhb* (complex II)) only *Ndufa10* was significantly upregulated in both groups. The other enzymes were only upregulated in the Mid PFNA group. Finally, expression of genes encoding enzymes involved in glucose metabolism were mainly disrupted by Mid PFNA + Mix, except for a few genes such as phosphofructokinase (*Pfkm*) that were only significant with Mid PFNA and carbonic anhydrase VII (*Ca7*) and STEAP family member 4 (*Steap4*), which were differentially expressed by both groups. Thus, the transcriptional profiles of Mid PFNA ± Mix are relatively similar with only subtle differences observed based on z-scores and p-values. Notably, a statistical comparison between Mid PFNA and Mid PFNA + Mix did not yield

Table 2 Genes related to energy homeostasis that were altered by Mid PFNA + Mix

Symbol	Entrez ID	Entrez gene name	$\log_2$(fold change)			Biological process
			Low PFNA + Mix	Mid PFNA	Mid PFNA + Mix	
Aco1	50655	Aconitase 1, soluble	0.12	0.337	0.544	Aerobic metabolism
Aco2	79250	Aconitase 2, mitochondrial	−0.065	0.428	0.448	Aerobic metabolism
Cox11	690300	Cytochrome c oxidase assembly homolog 11 (yeast)	0.24	0.593	0.235	Aerobic metabolism
Idh3b	94173	Isocitrate dehydrogenase 3 (NAD +) beta	0.003	0.325	0.247	Aerobic metabolism
Ndufa10	678759|316632	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 10, 42 kDa	0.042	0.3	0.275	Aerobic metabolism
Ndufab1	293453	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8 kDa	0.051	0.269	0.116	Aerobic metabolism
Ndufs3	295923	NADH dehydrogenase (ubiquinone) Fe–S protein 3, 30 kDa (NADH-coenzyme Q reductase)	0.085	0.336	0.28	Aerobic metabolism
Sdhb	298596	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	0.043	0.244	0.17	Aerobic metabolism
Sucla2	361071	Succinate-CoA ligase, ADP-forming, beta subunit	0.145	0.43	0.357	Aerobic metabolism
Suclg1	114597	succinate-CoA ligase, alpha subunit	0.024	0.275	0.284	Aerobic metabolism
Acaa1	501072|24157	Acetyl-CoA acyltransferase 1	0.113	1.008	0.985	Lipid metabolism
Acaa2	170465	Acetyl-CoA acyltransferase 2	0.394	0.556	0.969	Lipid metabolism
Acot1	314304	Acyl-CoA thioesterase 1	0.386	0.984	1.427	Lipid metabolism
Acot12	170570	Acyl-CoA thioesterase 12	0.245	0.557	0.666	Lipid metabolism
Acot2	192272	Acyl-CoA thioesterase 2	0.241	1.312	1.497	Lipid metabolism
Acot4	681337	Acyl-CoA thioesterase 4	0.264	1.115	1.585	Lipid metabolism
Acot7	26759	Acyl-CoA thioesterase 7	0.019	0.431	0.593	Lipid metabolism
Aldh1a1	24188	Aldehyde dehydrogenase 1 family, member A1	0.377	2.077	2.523	Lipid metabolism
Cyp2b6	361523|24300	Cytochrome P450, family 2, subfamily B, polypeptide 6	0.971	2.12	2.561	Lipid metabolism
Cyp2c19	293989	Cytochrome P450, family 2, subfamily C, polypeptide 19	0.128	0.686	0.756	Lipid metabolism
Cyp2j2	65210	Cytochrome P450, family 2, subfamily J, polypeptide 2	0.237	1.018	1.187	Lipid metabolism
Cyp4a11	50549	Cytochrome P450, family 4, subfamily A, polypeptide 11	0.643	2.419	2.568	Lipid metabolism
Cyp4a14	298423|24306	Cytochrome P450, family 4, subfamily a, polypeptide 14	0.333	1.201	1.335	Lipid metabolism
Decr1	117543	2,4-Dienoyl CoA reductase 1, mitochondrial	0.234	1.364	1.344	Lipid metabolism
Ephx2	65030	Epoxide hydrolase 2, cytoplasmic	0.243	1.201	1.267	Lipid metabolism
Gcdh	364975	Glutaryl-CoA dehydrogenase	0.175	0.576	0.638	Lipid metabolism
Hadh	113965	Hydroxyacyl-CoA dehydrogenase	0.056	0.418	0.4	Lipid metabolism
Hadha	170670	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), alpha subunit	0.144	0.659	0.746	Lipid metabolism
Hadhb	171155	Hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase (trifunctional protein), beta subunit	0.194	0.8	0.817	Lipid metabolism

Table 2 continued

Symbol	Entrez ID	Entrez gene name	Log$_2$(fold change)			Biological process
			Low PFNA + Mix	Mid PFNA	Mid PFNA + Mix	
Hsd11b1	25116	Hydroxysteroid (11-beta) dehydrogenase 1	−0.261	−0.446	**−1.082**	Lipid metabolism
Immt	312444	Inner membrane protein, mitochondrial	−0.011	**0.345**	**0.376**	Lipid metabolism
Mlycd	85239	Malonyl-CoA decarboxylase	0.093	**0.316**	**0.325**	Lipid metabolism
Ncaml	24586	Neural cell adhesion molecule 1	−0.163	−0.05	**−0.27**	Lipid metabolism
Phgdh	58835	Phosphoglycerate dehydrogenase	−0.308	**−1.271**	**−0.976**	Lipid metabolism
Plin5	501283	Perilipin 5	0.198	**0.595**	**0.61**	Lipid metabolism
Ptk2b	50646	Protein tyrosine kinase 2 beta	0.175	**0.607**	**0.47**	Lipid metabolism
Sirt4	304539	Sirtuin 4	0.162	0.291	**0.461**	Lipid metabolism
Acad11	315973	Acyl-CoA dehydrogenase family, member 11	0.115	**0.62**	**0.656**	Lipid metabolism—mitochondrial b-oxidation
Acadl	25287	Acyl-CoA dehydrogenase, long chain	0.05	**0.442**	**0.419**	Lipid metabolism—mitochondrial b-oxidation
Acadm	24158	Acyl-CoA dehydrogenase, C-4 to C-12 straight chain	0.15	**0.47**	**0.47**	Lipid metabolism—mitochondrial b-oxidation
Acads	64304	Acyl-CoA dehydrogenase, C-2 to C-3 short chain	0.056	**0.326**	**0.416**	Lipid metabolism—mitochondrial b-oxidation
Acadvl	25363	Acyl-CoA dehydrogenase, very long chain	0.047	**0.413**	**0.482**	Lipid metabolism—mitochondrial b-oxidation
Cpt2	25413	Carnitine palmitoyltransferase 2	0.163	**0.565**	**0.786**	Lipid metabolism—mitochondrial b-oxidation
Slc25a20	117035	Solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	0.098	**0.668**	**0.692**	Lipid metabolism—mitochondrial b-oxidation
Acox1	50681	Acyl-CoA oxidase 1, palmitoyl	0.074	**0.601**	**0.745**	Lipid metabolism—proxisomal b-oxidation
Crat	311849	Carnitine O-acetyltransferase	0.372	**1.55**	**1.71**	Lipid metabolism—proxisomal b-oxidation
Crot	83842	Carnitine O-octanoyltransferase	0.19	**1.115**	**1.219**	Lipid metabolism—proxisomal b-oxidation
Decr2	64461	2,4-Dienoyl CoA reductase 2, peroxisomal	0.275	**0.831**	**0.944**	Lipid metabolism—proxisomal b-oxidation
Ech1	64526	Enoyl CoA hydratase 1, peroxisomal	0.459	**1.503**	**1.856**	Lipid metabolism—proxisomal b-oxidation
Eci2	291075	Enoyl-CoA delta isomerase 2	0.117	**0.577**	**0.548**	Lipid metabolism—proxisomal b-oxidation
Ehhadh	171142	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	0.184	**1.702**	**1.86**	Lipid metabolism—proxisomal b-oxidation
Hsd17b4	79244	Hydroxysteroid (17-beta) dehydrogenase 4	0.113	0.295	0.278	Lipid metabolism—proxisomal b-oxidation
Apoa2	25649	Apolipoprotein A-II	0.321	**0.504**	*0.474*	Lipid metabolism—transport
Abcd3	25270	ATP-binding cassette, sub-family D (ALD), member 3	0.093	**0.729**	**0.685**	Lipid metabolism—transport
Cd36	29184	CD36 molecule (thrombospondin receptor)	0.158	**1.276**	**1.867**	Lipid metabolism—transport
Fabp5	140868	Lipid binding protein 5 (psoriasis-associated)	−0.385	**−1.392**	**−1.508**	Lipid metabolism—transport
Slc27a2	65192	Solute carrier family 27 (Lipid transporter), member 2	0.215	**0.543**	**0.57**	Lipid metabolism—transport
Adrb3	25645	Adrenoceptor beta 3	−0.279	−0.134	**−0.366**	Glucose metabolism disorders
Ca1	310218	Carbonic anhydrase I	−0.003	−0.78	**−1.132**	Glucose metabolism disorders
Ca4	29242	Carbonic anhydrase IV	−0.266	−0.009	**−0.236**	Glucose metabolism disorders
Ca7	291819	Carbonic anhydrase VII	−0.06	**0.817**	**0.643**	Glucose metabolism disorders
Calcr	116506	Calcitonin receptor	0.225	0.114	**0.392**	Glucose metabolism disorders

Table 2 continued

| Symbol | Entrez ID | Entrez gene name | Log$_2$(fold change) | | | Biological process |
			Low PFNA + Mix	Mid PFNA	Mid PFNA + Mix	
Dnajc3	63880	DnaJ (Hsp40) homolog, subfamily C, member 3	−0.113	**−0.279**	**−0.362**	Glucose metabolism disorders
Fbp2	114508	Fructose-1,6-bisphosphatase 2	0.272	*0.642*	**1.081**	Glucose metabolism disorders
Hdc	24443	Histidine decarboxylase	0.267	1.03	**1.309**	Glucose metabolism disorders
Hipk1	365895	Homeodomain interacting protein kinase 1	0.189	0.011	**0.296**	Glucose metabolism disorders
Lef1	161452	Lymphoid enhancer-binding factor 1	0.1	0.36	**0.573**	Glucose metabolism disorders
Mgat2	94273	Mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase	−0.135	*−0.436*	**−0.544**	Glucose metabolism disorders
Pfkm	65152	Phosphofructokinase, muscle	−0.388	**−0.487**	−0.35	Glucose metabolism disorders
Prox1	305066	Prospero homeobox 1	−0.09	−0.114	**−0.336**	Glucose metabolism disorders
Psmc3	29677	Proteasome (prosome, macropain) 26S subunit, ATPase, 3	0.102	*0.287*	**0.326**	Glucose metabolism disorders
Psmd8	292766	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 8	0.103	0.179	**0.238**	Glucose metabolism disorders
Rampl	58965	Receptor (G protein-coupled) activity modifying protein 1	−0.133	−0.213	**−0.299**	Glucose metabolism disorders
Rt1-Ba	309621	Major histocompatibility complex, class II, DQ alpha 1	*−0.244*	−0.139	**−0.302**	Glucose metabolism disorders
Steap4	499991	STEAP family member 4	−0.15	**−0.332**	**−0.404**	Glucose metabolism disorders
Hmgcs2	24450	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	0.118	**0.469**	**0.471**	Ketogenesis
Pex11a	85249	Peroxisomal biogenesis factor 11 alpha	0.209	**1.368**	**1.387**	Peroxisome biogenesis
Pex16	311203	Peroxisomal biogenesis factor 16	0.208	**0.405**	**0.439**	Peroxisome biogenesis
Pex5	312703	Peroxisomal biogenesis factor 5	0.077	**0.326**	*0.284*	Peroxisome biogenesis
Dhrs4	266686	Dehydrogenase/reductase (SDR family) member 4	0.005	**0.363**	*0.294*	Retinol metabolism

Roman non-significant, *italic p* = 0.05–0.1, *bold p* < 0.05

Table 3 Upstream regulators predicted to regulate effects caused by Mid-PFNA ± Mix

Upstream regulator	Mid PFNA + Mix		Mid PFNA		Genes
	z-score	p-value	z-score	p-value	
PPARα Peroxisome proliferator-activated receptor α	5.3	2e–24	5.1	5e–28	Abcd3, Acaa1, Acaa2[a], Acadl, Acadm, Acads, Acadvl, Acat1, Acot1[a], Acot2, Acox1, Adtrp, Cd36, Cpt2, Crot, Cyp2b6, Cyp4a11, Cyp4a14, Decr1, Decr2, Ech1, Eci2, Ehhadh, Fabp5, Gpd1, Hadh, Hadha, Hadhb, Rt1-Ba[a], Hmgcs2, Mlycd, Pex11a, Plin5, Slc25a20, Slc27a2, Vnn1, Aadac[b], Apoa2[b], Cfh[b], Ftcd[b], H2afz[b]
KLF15 Krüppel-like factor 5	3.2	3e–18	3.0	5e–17	Acadl, Acadm, Acadvl, Acot1[a], Acox1, Cd36, Cpt2, Decr1, Ehhadh, Fabp5, Hadha, Hadhb, Mlycd, Slc25a20
ACOX1 ↑Peroxisomal acyl-coenzyme A oxidase 1	−2.4	5e–15	−2.474	3e–13	Abcd3, Acaa1, Acadl, Acadm, Acadvl, Acot2, Acox1, Aig1, Cd36, Crat, Cyp4a11, Cyp4a14, Ehhadh, Rt1-Ba[a], Hsd11b1[a], Pex11a, Slc27a2, Tnfrsf10a[a], Hspa5[b]
PPARγ Peroxisome proliferator-activated receptor α	3.9	8e–14	3.3	1e–13	Acaa1, Acaa2[a], Acadl, Acadm, Acads, Acox1, Adrb3[a], Cd36, Cpt2, Crat, Cyp4a11, Cyp4a14, Ehhadh, Fabp5, Fbp2[a], Gpd1, Hadha, Hadhb, Hmgcs2, Mlycd, Pepd, Pex11a, Plin5, Slc25a20, Vnn1, Apoa2[b], Sdc1[b]
EHHADH ↑Enoyl-coenzyme A, hydratase/3-hydroxyacyl coenzyme A dehydrogenase	−2.8	1e–12	−2.8	6e–13	Abcd3, Acaa1, Acot2, Acox1, Cd36, Cyp4a11, Cyp4a14, Pex11a
HSD17B4 ↑Peroxisomal multifunctional enzyme type 2	−3.0	1e–12	−2.8	4e–11	Abcd3, Acaa1, Acot2, Acox1, Cd36, Cyp4a11, Cyp4a14, Hoxd13[a], Pex11a

An arrow indicating up- or downregulation marks upstream regulators that are differentially expressed genes. Negative z-scores indicate that the upstream regulator is inactivated, whereas positive z-scores indicate activation

[a] DEG only present in Mid PFNA + Mix

[b] DEG only present in Mid PFNA

any differentially expressed genes, hence further studies with larger sample sizes are required to verify some of the subtle differences indicated by this study.

The IPA has a function to predict the activity of potential upstream regulators based on differentially expressed genes. Using our dataset, we could predict an increased activity of several transcription factors and other central proteins in livers from exposed animals (Table 3). Downstream of the differentially expressed genes, IPA predicts regulatory effects such as diseases and functions likely to be perturbed due to the changes in gene expression. As illustrated in Fig. 4, activation of the upstream regulators PPARα, PPARγ, PPARδ, RXRα, PPARgc1a, and MED1 results in altered gene expression (Table 2) that subsequently leads to increased oxidation of lipids, in particular fatty acids. This, in turn, protects against accumulation of lipids in the liver and therefore also against hepatic steatosis. This is consistent with our observations in livers from rats having been exposed to Low- and Mid PFNA doses, with no indications of lipid accumulation, increased cell size, or compromised cell borders (Hadrup et al. 2015). However, as exposure to high PFNA doses causes adverse effects such as steatosis (Hadrup et al. 2015), it can be speculated that livers from at least the Mid

PFNA exposed rats could become affected over time. The fact that hepatic steatosis was evident at the high PFNA dose (5 mg/kg/day) and that signs of protection against hepatic steatosis was observed at the mid PFNA dose (0.25 mg/kg/day) illustrates the problem of extrapolating from toxicological observations at higher doses to effects at lower doses, which is common practice in risk assessment of chemicals.

PPARs are known regulators of fatty acid β-oxidation (Kanehisa and Goto 2000), and increased β-oxidation results in decreased plasma lipid concentration (Lau 2012). Then, as perfluorinated alkyl acids are known PPAR-activators (Vanden Heuvel et al. 2006; Lau 2012; U. S. Environmental Protection Agency 2014), effects observed in livers from the PFNA groups could, at least in part, be explained by this regulatory pathway. Further, the Mix contains two phthalates, DBP and DEHP, which are both known PPAR activators (Desvergne et al. 2009), potentially adding to the effect in the PFNA + Mix groups. In fact, additional compounds in the Mix such as butyl-paraben, linuron, and vinclozolin, can stimulate PPAR-dependent transcription (U. S. Environmental Protection Agency 2014), further adding to the overall effect. However, at the doses used in our study, the contribution of Mix
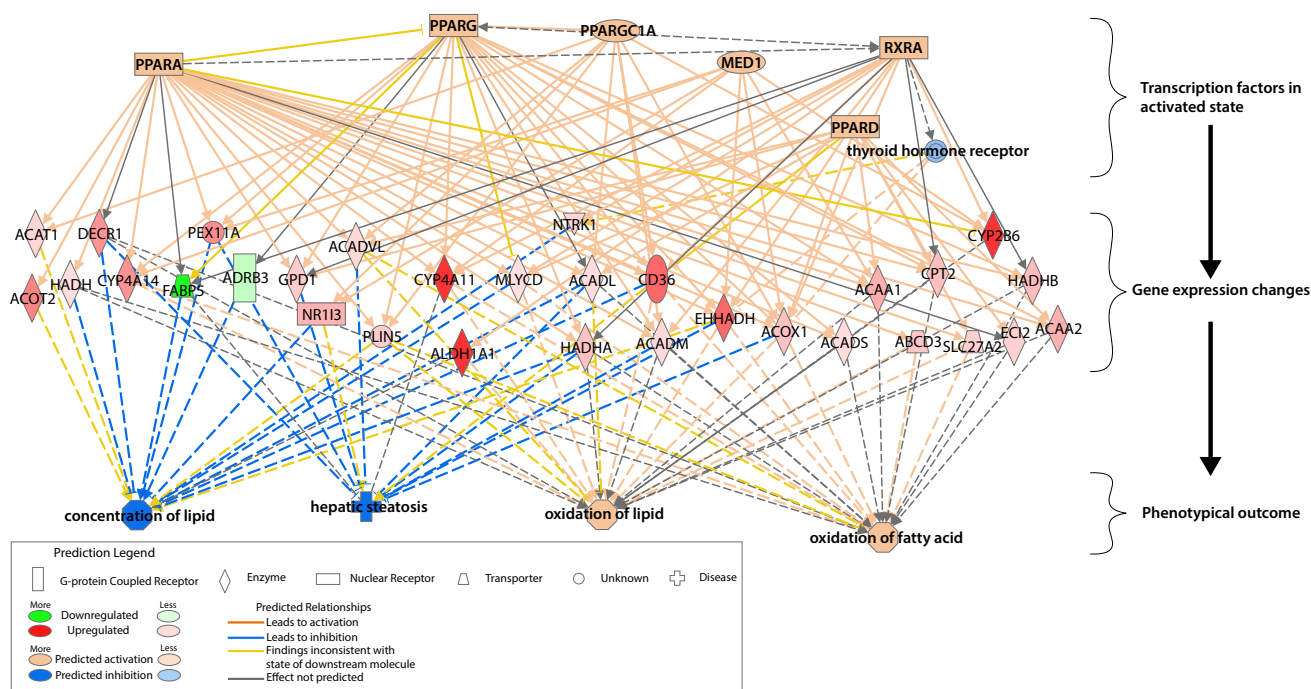
Fig. 4 Illustration of a putative hepatic signaling network, from PPAR (and other transcriptional regulators) activation through regulation of gene expression to ultimately dictate phenotypic outcomes. *Orange* indicates predicted activation and *blue* indicates predicted inhibition.

Shades of *red* and *green* indicate level of increased and decreased gene expression, respectively. The network was generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) (Color figure online)

towards PPAR activation is likely minor since (i) we observed very small differences in transcript levels between the Mid PFNA and Mid PFNA + Mix groups, and (ii) we did not observe any significant changes in gene expression in animals exposed to Low PFNA + Mix.

A summary of the functions of the Mid PFNA ± Mix is given in Supplementary Table 4, together with genes that displayed highest fold-change difference in transcript levels between Mid PFNA + Mix and Mid PFNA alone. As discussed, altered CYP2C9 activity might play a role in the changed plasma lipid concentrations. However, our data do not suggest downregulation of the rat CYP2C9 homologue, *Cyp2c11*. On the other hand, the HDL-constituent, apolipoprotein A-II (encoded by *Apoa2*) is upregulated by PFNA but not by PFNA + Mix. The effects observed for Mix and PFNA + Mix might therefore be caused by a combination of decreased CYP2C9 activity and PPAR activation.

4 Concluding remarks

The characterization of potential toxic effects caused by prolonged exposure to various compounds can be a challenge. When a large number of compounds are present at the same time, as in humans, the challenge is even greater.

In an effort to better understand the complex interaction between multiple exogenous compounds and the living organism, we profiled the plasma metabolome and the liver transcriptome in rats after exposure to a low dose of bioavailable chemicals. We observed that a mixture of 14 chemicals at high-end human exposure levels (Mix), low- to mid-range doses of PFNA, or a combination of the two, had the potential to alter the blood plasma levels of diacylglycerols, PCs, and cholesterol derivatives, mimicking some of the features of metabolic syndrome. These effects can also suggest an inflammatory response, but in either case warrant further investigations. We also observed more subtle changes to the liver transcriptome following Mid PFNA exposure irrespective of Mix, suggesting that the hepatic effects were driven by PFNA. In conclusion, this study demonstrates that low-dose exposure to chemical mixtures can affect the metabolome and cause disturbed lipid homeostasis, and that 'omics' approaches are powerful tools to detect smaller changes not readily observable at the macroscopic level.

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