Proteomics and metabolic phenotyping define principal roles for the aryl hydrocarbon receptor in mouse liver

Jian Jina,b, Banrida Wahlangc,d, Monika Thapae, Kimberly Z. Headc, Josiah E. Hardestyc, Sudhir Srivastavaf,g, Michael L. Merchantb,h,i,j, Shesh N. Raid,c,h,j,k,l, Russell A. Proughe, Matthew C. Cavea,c,d,e,h,j,k,*

aDepartment of Pharmacology & Toxicology, the University of Louisville School of Medicine, Louisville, KY 40202, USA
bDepartment of Endocrinology, the Second Affiliated Hospital & Wuying Children’s Hospital, Wenzhou Medical University, Wenzhou 325027, China
cDivision of Gastroenterology, Hepatology & Nutrition, Department of Medicine, the University of Louisville School of Medicine, Louisville, KY 40202, USA
dSuperfund Research Center, the University of Louisville, Louisville, KY 40202, USA
eDepartment of Biochemistry and Molecular Genetics, the University of Louisville School of Medicine, Louisville, KY 40202, USA
fDepartment of Bioinformatics and Biostatistics, the School of Public Health and Information Sciences, the University of Louisville, Louisville, KY 40202, USA
gCentre for Agricultural Bioinformatics, ICAR-Indian Agricultural Statistics Research Institute, New Delhi 110012, India
hThe Center for Integrative Environmental Health Sciences, University of Louisville, Louisville, KY 40202, USA
iDivision of Nephrology & Hypertension, Department of Medicine, the University of Louisville School of Medicine, Louisville, KY 40202, USA
jThe Hepatobiology and Toxicology Center, University of Louisville, Louisville, KY 40202, USA
kAlcohol Research Center, University of Louisville, Louisville, KY 40202, USA
lBiostatistics and Bioinformatics Facility, James Graham Brown Cancer Center, Louisville, KY 40202, USA

Received 23 July 2021; received in revised form 23 September 2021; accepted 28 September 2021

Abbreviations: AHR, aryl hydrocarbon receptor; ALT, alanine transaminase; ANOVA, analysis of variance; AST, aspartate transaminase; AUC, area under the curve; CAR, constitutive androstane receptor; CD36, cluster of differentiation 36; CYP, cytochrome P450; EPF, enrichment by protein function; FDR, false discovery rate; FGF21, fibroblast growth factor 21; IGFB1, insulin-like growth factor 1; IL-6, interleukin 6; GCR, glucocorticoid receptor; GO, gene ontology; H&E, hematoxylin-eosin; HDL, high-density lipoprotein; HFD, high fat diet; IFP, interaction by protein function; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein-1; mIR, microRNA; MUF, major urinary protein; NAFLD, non-alcoholic fatty liver disease; nHDLc, non-HDL cholesterol; NFKBIA, nuclear factor kappa-inhibitor alpha; PAI-1, plasminogen activator inhibitor-1; PCB, polychlorinated biphenyl; PLIN2, perilipin-2; PNPLA3, patatin-like phospholipase domain-containing protein 3; PPAR, peroxisome proliferator-activated receptor; SGK1, serum/glucocorticoid regulated kinase; TAF1LD, toxicant-associated fatty liver disease; TASH, toxicant-associated steatohepatitis; TAT, tyrosine aminotransferase; TMT, tandem mass tag; VLDL, very low-density lipoprotein; WT, wild type; ZFP125, zinc finger protein 125.
*Corresponding author. Tel.: +1 502 8525252; fax: +1 502 8528927.
E-mail address: matt.cave@louisville.edu (Matthew C. Cave).

Peer review under responsibility of Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences

https://doi.org/10.1016/j.apsb.2021.10.014

2211-3835 © 2021 Chinese Pharmaceutical Association and Institute of Materia Medica, Chinese Academy of Medical Sciences. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
1. Introduction

The liver is the largest internal organ. It is responsible for myriad detoxification and synthetic processes which protect and nourish the body. Likely due to its prominent roles in xenobiotic and intermediary metabolism, the liver is the principal target of toxicities from alcohol, pharmaceuticals, environmental pollutants and obesity. The liver-related death rates from cirrhosis and liver cancer are rapidly increasing\(^1\). Nonalcoholic fatty liver disease (NAFLD) is the most prevalent liver disease worldwide, and its more severe form is called non-alcoholic steatohepatitis. Although typically considered to be the hepatic manifestation of obesity and metabolic syndrome, NAFLD may also be caused or modulated by pharmaceuticals and environmental pollutants including some endocrine and metabolism disrupting chemicals\(^2\)–\(^6\). The terms toxicant-associated fatty liver disease (TAFLD) and toxicant-associated steatohepatitis (TASH) have been proposed to describe the latter situation\(^2,7\).

Many liver physiologic and pathophysiologic processes are regulated by ligand-activated transcription factors, including the aryl hydrocarbon receptor (AHR). The AHR is a well-established master regulator of xenobiotic metabolism. Its canonical target genes include the cytochrome P4501A (Cyp1a) family, also implicated in carcinogenesis\(^8\). AHR’s high-affinity binding ligands include dioxins (e.g., 2,3,7,8-tetrachlorodibenzo-p-dioxin) and other dioxin-like molecules (e.g., 3,3′,4,4′,5-pentachlorobiphenyl also called polychlorinated biphenyl 126 or PCB126). The high thermodynamic stability of these persistent organic pollutants makes them resistant to degradation leading to sustained AHR activation. AHR’s low-affinity ligands include rapidly metabolized dietary/endogenous molecules such as: flavonoids, bilirubin, and gut microbiome-derived tryptophan metabolites\(^9\). Apart from its regulatory role in hepatic xenobiotic metabolism and carcinogenesis, the AHR has also been implicated in intermediary metabolism\(^10\), metal homeostasis\(^11,12\), fibrosis\(^13\) and the production of hepatokines and liver-derived circulating proatherogenic molecules\(^14,15\). Given AHR’s role in the regulation of intermediary metabolism, it is not surprising that dioxins and dioxin-like molecules have been associated with endocrine and metabolic disruption\(^15\). Our group and others have been studying these foreign compounds in TAFLD/TASH\(^3,5,12,14,16–19\).

The role of the AHR in hepatic lipid metabolism and obesity-related diseases like NAFLD is complex. Complicating matters, this role may be ligand-dependent and species/strain-dependent\(^2\). While some dioxins and dioxin-like molecules may cause TAFLD, some endogenously-produced AHR ligands may be protective against at least some aspects liver disease\(^13\). Moreover, while some strains of Ahr\(^−/−\) mice spontaneously developed steatosis, others were protected against diet-induced fatty liver disease\(^20–24\). While a proteomics approach has previously been utilized to investigate PCBs in TAFLD\(^16\), we could find no liver proteomics data for Ahr knockout mice in the published literature. Therefore, in this manuscript we performed paired liver proteomics and metabolic phenotyping in wild type and whole-body Ahr knockout mice with or without PCB126-induced ligand-activation of the AHR. The top liver pathways associated with these interventions were elucidated. The results will enhance the current understanding of AHR’s principal roles in liver health and metabolic diseases.

2. Materials and methods

2.1. Animal studies

The animal protocol was approved by the University of Louisville Institutional Animal Care and Use Committee (Louisville, KY, USA). Adult male C57BL/6 mice (wild type, WT, catalog number: B6-M, 9 weeks old) and Ahr\(^−/−\) mice (catalog number: 9166-M, 8–9 weeks old) were purchased from Taconic Biosciences Laboratory (Hudson, NY, USA). While additional strains of Ahr\(^−/−\) mice have been generated by other laboratories, this strain was chosen because it is a whole-body knockout model

**Abstract**  Dioxin-like molecules have been associated with endocrine disruption and liver disease. To better understand aryl hydrocarbon receptor (AHR) biology, metabolic phenotyping and liver proteomics were performed in mice following ligand-activation or whole-body genetic ablation of this receptor. Male wild type (WT) and Ahr\(^−/−\) mice (Taconic) were fed a control diet and exposed to 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) (61 nmol/kg by gavage) or vehicle for two weeks. PCB126 increased expression ofcanonical AHR targets (Cyp1a1 and Cyp1a2) in WT but not Ahr\(^−/−\). Knockouts had increased adiposity with decreased glucose tolerance; smaller livers with increased steatosis and perilipin-2; and paradoxically decreased blood lipids. PCB126 was associated with increased hepatic triacylglycerides in Ahr\(^−/−\). The liver proteome was impacted more so by Ahr\(^−/−\) genotype than ligand-activation, but top gene ontology (GO) processes were similar. The PCB126-associated liver proteome was Ahr-dependent. Ahr principally regulated liver metabolism (e.g., lipids, xenobiotics, organic acids) and bioenergetics, but it also impacted liver endocrine response (e.g., the insulin receptor) and function, including the production of steroids, hepatokines, and pheromone binding proteins. These effects could have been indirectly mediated by interacting transcription factors or microRNAs. The biologic roles of the AHR and its ligands warrant more research in liver metabolic health and disease.
generated on a C57BL/6 background, and it was commercially available. Importantly, C57BL/6 mice have a high-affinity AHR. Taconic specifically recommend the B6-M WT strain as their most appropriate control for the 9166-M knockout model, although B6-M is not a litter-mate control. Notably, our co-authoring toxicologist, Dr. Wahlang, previously published on the hepatotoxicity of PCB126 in Taconic’s B6-M WT strain, albeit at a higher PCB126 dose and different diet. That manuscript documented induction of the canonical AHR target gene, Cyp1a1, and metabolic disruption by PCB126.

All mice were fed a control synthetic diet (20.0%, 69.8%, and 10.2% of total calories from protein, carbohydrate, and fat; TekLad TD06416). At 9–10 weeks of age, the mice were administered either vehicle control (corn oil) or 20 μg/kg PCB126 (61 mmol/kg) via a one-time gavage for two weeks. The PCB126 dose and duration of exposure is justified in our prior publications. Briefly, this dose is lower than that typically used by other investigators, and we believe it to be relevant to human exposures while still activating key canonical AHR target genes, Cyp1a1 and Cyp1a2. These procedures generated four groups of mice (n = 10 mice) which were designated as: the WT Vehicle group; WT PCB126 group; the Ahr−/− Vehicle group and the Ahr−/− PCB126 group. The study design is summarized graphically in Supporting Information Fig. S1. After two weeks, the mice were fasted overnight and dual energy X-ray absorptiometry scanning (Lunar PIXimus densitometer, WI, USA) was performed to analyze body composition. Tissues (e.g., liver, adipose, plasma, etc.) were then collected following administration of ketamine/xylazine (120/16 mg/kg body weight) given by intraperitoneal injection. Plasma was collected with EDTA as the anticoagulant.

2.2. Histological staining

Liver and adipose tissues were fixed in 10% neutral buffered formalin for 72 h and embedded in paraffin for routine histological examination. Hematoxylin-eosin (H&E) staining was performed to identify histopathological changes. In order to better evaluate histologic steatosis, Oil Red O stain was done in tissue that was placed in optimal cutting temperature reagent at the time of examination. Hematoxylin-eosin (H&E) staining was performed to identify histopathological changes. In order to better evaluate histologic steatosis, Oil Red O stain was done in tissue that was placed in optimal cutting temperature reagent at the time of necropsy and snapped frozen in liquid nitrogen. Micrographic images were acquired by a high-resolution digital scanner (Olympus) with a digital camera (Olympus BX41).

2.3. Real-time PCR

Mouse liver tissues were homogenized and total RNA was extracted using RNA-STAT 60 (AMSBIO, Cambridge, MA, USA) according to the manufacturer’s protocol. The purity and quantity of total RNA were assessed with a Nanodrop spectrometer (ND-1000, ThermoFisher Scientific, Waltham, MA, USA) using ND-1000 V3.8.1 software. cDNA was reverse transcribed from 1 μg RNA with a one-step cDNA synthesis reagent (QuantiTect cDNA Supermix, QuantaBio, Beverly, MA, USA). Then RT-PCR was performed on the CFX384TM Real-Time System (BioRad, Hercules, CA, USA) using iTaq Universal probe Supermix and Taqman probes as described previously. All reactions were performed in triplicate. The relative mRNA expression was calculated using the comparative 2−ΔΔCt method and normalized against GAPDH mRNA. MicroRNAs (miRs) were isolated from mouse livers using the MagMAX™ mirVana™ Total RNA Isolation Kit (ThermoFisher Scientific) according to the manufacturer’s protocol. Hepatic expression for selected miRs was measured using RT-PCR, like the gene expression (mRNA) method but using Taqman miR probes (ThermoFisher Scientific).

2.4. Measurement of hepatic lipids, plasma lipids and cytokines

The liver tissues were rinsed in 1× phosphate buffered saline and homogenized in 50 mmol/L NaCl solution. Hepatic lipids were extracted by a mixed solution of chloroform and methanol (2:1) according to a published protocol. Hepatic triglycerides and free fatty acids were assessed using commercial kits with final values normalized to liver weight. Plasma alanine transaminase (ALT), aspartate transaminase (AST), cholesterol, triglyceride, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL), and non-HDL cholesterol (nHDLc) levels were determined with lipid panel plus kits on a Piccolo Xpress Chemistry Analyzer (Abbott Laboratories, Chicago, IL, USA). Plasma cytokine and adipokine levels were evaluated using a customized Milliplex MAP mouse adipokine panel (Millipore Sigma, Billerica, MA, USA) on a Luminex 100 system (Luminex Corp, Austin, TX, USA).

2.5. Proteomics analysis

Proteins were extracted from liver tissues in 1% SDS modified RIPA buffer with protease and phosphatase inhibitors, using a bead homogenizer, and protein amounts were measured by BCA protein assay. Protein lysates (200 μg) were trypsinized using the modified filter-aided sample preparation method. Protein samples were first reduced by dithiothreitol, denatured by 8 mol/L urea and alkylated by iodoacetamide, followed by centrifugation through a high molecular weight cutoff centrifugal filter (Millipore Sigma, 10k MWCO). Next, after overnight digestion with sequencing grade trypsin (Promega, Madison, WI, USA) at 37 °C, the digested peptides were collected and cleaned with a C18 Proto™ 300 Å ultra microspin column. Digested peptide samples (50 μg) were labeled with tandem mass tag (TMT) TMT10plex™ isobaric label reagent set (ThermoFisher Scientific). Next, they were concentrated and desalted with Oasis HLB extraction cartridges (Waters Corporation, Milford, MA, USA) using a modified protocol for extraction of the digested peptides. Samples were separated by high pH reversed phase separation with fraction concatenation on a Beckman System Gold LC system supplemented with 126 solvent module and 166 UV—Vis detector in tandem with a BioRad Model 2110 Fraction Collector.

Liquid chromatography/mass spectrometry was used to measure TMT-labeled peptides. Briefly, every high pH reversed phase fraction was dissolved in 50 μL solution of the combination of 2% v/v acetonitrile with 0.1% v/v formic acid. 1 μL of each fraction was analyzed on EASY-nLC 1000 UHPLC system (Thermo Fisher Scientific) and an Orbitrap Elite—ETD mass spectrometer (Thermo Fisher Scientific). The raw data from the mass spectrometer were analyzed by Proteome Discoverer v2.2.0.388.

2.6. Statistical analysis and data sharing

Statistical evaluation was performed by two-way analysis of variance (ANOVA) using GraphPad Prism version 7.02 for Windows (GraphPad Software Inc., La Jolla, CA, USA), and Tukey’s test was used as a post hoc test. The two factors analyzed were mouse genotype (genotype effect) and PCB126 exposure (PCB effect). An interaction effect was also determined. Results are
reported as mean ± standard deviation (SD). P < 0.05 was considered statistically significant.

Statistical analysis for the proteomic data was carried out with R packages using a modified version of our biostatistician’s previously published protocol. First, the raw data were transformed by taking logarithmic base 2 followed by quantile normalization. Then, the missing values were imputed using singular value decomposition method. Proteins with missing values > 40% were excluded from subsequent analysis. Finally, differentially abundant proteins (P < 0.05) were further filtered by fold-change (FC) criteria (−1 < log₂FC < 1) and multiple comparisons testing with a false discovery rate (FDR) <0.05 yielding the final significant results for the differentially abundant proteins. Next, these proteins were imported into MetaCore software (Clarivate Analytics, Philadelphia, PA, USA) and the following analyses were performed: gene ontology (GO) processes, enrichment by protein function (EPF), and interaction by protein function (IPF). Proteomics data files were deposited with MassIVE (http://massive.ucsd.edu) data repository, Center for Computational Mass Spectrometry at the University of California, San Diego and shared with the ProteomeXchange (www.proteomexchange.org).

3. Results

3.1. Impact of Ahr genotype and PCB126 exposure on the hepatic expression of key xenobiotic receptors and their target genes

The mRNA expression levels of Ahr, Pxr and Car, as well as their target genes were assessed by RT-PCR. These studies confirmed the absence of Ahr expression in the knockout mice (Fig. 1A). Consistent with this finding, expression of the canonical AHR target genes, Cyp1al and Cyp1a2, was significantly decreased in Ahr−/− (genotype effect, Fig. 1B and C). In WT mice, PCB126 exposure increased Cyp1al (~2610-fold) and Cyp1a2 (~10-fold) expression vs. vehicle consistent with AHR activation. Ahr−/− mice had significantly increased mRNA expression of Pxr (genotype effect, Fig. 1D) and its target gene, Cyp3a11 (genotype effect, Fig. 1E). Car expression was down-regulated in Ahr−/− mice and up-regulated by PCB126 exposure (genotype effect and PCB effect, Fig. 1F). However, expression of the CAR target gene, Cyp2b10, was paradoxically increased in Ahr−/− mice (genotype effect, Fig. 1G). Thus, Ahr−/− mice had deleted Ahr; increased Pxr expression and activity; and increased Car activity despite reduced Car expression. PCB126 activated the AHR, and to a lesser degree, CAR.

3.2. Impact of Ahr genotype and PCB126 exposure on body composition, blood glucose and lipids

Body weight and composition varied by experimental group. Body weights over time are given in Supporting Information Fig. S2A. The Ahr−/− mice had significantly lower end of study body weights (genotype effect, Fig. S2B). However, because these mice also weighed less at the start of the study (Fig. S2A), the % change in body weight did not differ between the Ahr−/− and WT mice (Fig. 2A). However, PCB126 exposure was associated with reduced % change in body weight (PCB effect, Fig. 2A). Body composition varied according to genotype. The total % body fat (genotype effect, Fig. 2B) and the white adipose tissue to body weight ratio (genotype effect, Fig. S2C) were significantly increased in Ahr−/− mice, while % lean body mass was reduced (genotype effect, Fig. 2C).

Regarding biomarkers of intermediary metabolism, Ahr−/− genotype was associated with significantly decreased fasting blood glucose (genotype effect, Fig. 2D) and insulin (genotype effect, Fig. 2E). Curves generated from the glucose tolerance test area under the curve (AUC) data are provided in Fig. 2F and G. PCB126 exposure was associated with improved glucose tolerance (PCB effect), with a trend towards worsened glucose tolerance in Ahr−/− mice (P = 0.16, genotype effect). However, AUC was significantly increased in Ahr−/− Vehicle vs. WT Vehicle and in Ahr−/− PCB126 vs. WT PCB126. Ahr−/− was associated with significantly reduced total cholesterol (genotype effect, Fig. 2H), HDL-c (genotype effect, Fig. 2I) and triglycerides (genotype effect, Fig. 2J) with a trend towards reduced VLDL (P = 0.06, genotype effect, Fig. S2D). These lipid parameters were not affected by PCB126. No significant differences were seen for LDL-c (Fig. S2E).

Additionally, histological analysis on white adipose sections was performed (Fig. S2F), and no differences were observed in adipocyte morphology between groups. In summary, Ahr−/− genotype was associated with increased adiposity and variably worsened glucose tolerance with paradoxically decreased fasting plasma glucose, insulin and lipid levels. PCB126 exposure was associated with decreased body weight gain and improved glucose tolerance.

3.3. Impact of Ahr genotype and PCB126 exposure on liver

Representative liver histology is provided in Fig. 3A (H&E stain) and Fig. 3B (Oil Red O stain). These images show qualitatively increased hepatic steatosis in the Ahr−/− mice. Consistent with the histology, hepatic triglycerides (genotype effect, Fig. 3C) and free fatty acids (genotype effect, Fig. 3D) were increased with Ahr ablation, although hepatic cholesterol was unchanged (Fig. 3E). PCB126 exposure was associated with increased hepatic triglycerides (PCB effect, Fig. 3C), but this effect was driven solely by the comparison of Ahr−/− PCB126 vs. Ahr−/− Vehicle. Despite increased steatosis, the liver to body weight ratio was reduced in Ahr−/− mice (genotype effect, Fig. 3F). Plasma ALT (genotype effect, Fig. 3G) and AST (genotype effect, Fig. 3H) activities were increased in the knockout mice. Hepatic mRNA expression of the scavenger receptor, cluster of differentiation 36 (Cd36), and the lipase, patatin like phospholipase domain containing protein 3 (Pnpa3), were measured. Cd36 (Fig. 3I) was increased by PCB126 exposure (PCB effect) and by Ahr−/− genotype (genotype effect). Pnpa3 was reduced in Ahr−/− (genotype effect, Fig. 3J). Overall, these data show that the Ahr−/− mice had increased liver steatosis and injury which could be related, in part, to increased blood lipid uptake by hepatic CD36 receptors.

3.4. Impact of Ahr genotype and PCB126 exposure on plasma adipocytokines and hepatokine mRNA expression

Despite having increased obesity and NAFLD, the Ahr−/− mice had decreased circulating levels of pro-inflammatory cytokines including: resistin (genotype effect, Supporting Information Fig. S3A) and interleukin 6 (IL-6, genotype effect, Fig. S3B). Plasma monocyte chemotactic protein-1 (MCP-1, Fig. S3C) and plasminogen activator inhibitor-1 (PAI-1, Fig. S3D) were unchanged. The adipokine, leptin, was increased in Ahr−/− mice (genotype effect, Fig. S3E) consistent with the increased % body fat in these mice. These adipocytokines were not changed by PCB126.

The mRNA expression of several hepatokines previously implicated in obesity, diabetes and NAFLD was determined. The
AHR suppressed fibroblast growth factor (Fgf21) transcription via binding to a xenobiotic response element within the Fgf21 promoter. Therefore, it was not surprising that Fgf21 expression was dramatically increased in \textit{Ahr}\textsuperscript{e/e} mice (genotype effect, Fig. S3F). Insulin-like growth factor 1 (Igf1) was slightly increased by PCB126 (PCB effect, Fig. S3G), while betatrophin was reduced by \textit{Ahr}\textsuperscript{e/e} (genotype effect, Fig. S3H) or PCB126 exposure (PCB effect, Fig. S3H). The observed increases in Fgf21 and Igf1 expression should theoretically mitigate obesity-associated diseases, while the reduced betatrophin could lead to decreased pancreatic beta cell mass and eventually worsened diabetes over time. In summary, PCB126 was associated with increased Igf1 and decreased betatrophin. 

3.5. Impact of Ahr genotype and PCB126 exposure on the hepatic proteome

3.5.1. Primary proteomics results

5075 unique proteins and their isoforms were detected. \textit{Ahr}\textsuperscript{e/e} and exposure to the AHR agonist, PCB 126, produced distinct hepatic proteomes (Supporting Information Table S1). To facilitate interpretation of intergroup comparisons, volcano plots and a Venn diagram were constructed demonstrating differential abundance of hepatic proteins (Supporting Information Fig. S4).

In WT mice, PCB126 exposure (vs. vehicle control) was associated with 8 differentially abundant proteins (5 increased and 3 decreased, Fig. 4A). PCB126 increased hepatic abundance of CYP1A1 (Log\textsubscript{2}FC = 4.82) and CYP1A2 (Log\textsubscript{2}FC = 4.36) consistent with the mRNA expression data (Fig. 1B and C). These P450s were the proteins with the largest fold-changes and greatest statistical significances in this intergroup comparison.

\textit{Ahr}\textsuperscript{e/e} vs. WT genotype was associated with 340 differentially abundant proteins (279 increased and 61 decreased) in vehicle-administered animals (Fig. 4B). Cyp1A1 protein (Log\textsubscript{2}FC = 3.61) was increased in \textit{Ahr}\textsuperscript{e/e} consistent with its mRNA expression (Fig. 1E). Cyp1A2 was increased at the protein level (Log\textsubscript{2}FC = 1.91) but not the mRNA level (Fig. 1C). The up-regulated proteins with either the greatest fold-change or statistical significance included: glutathione S-transferase A1 (Log\textsubscript{2}FC = 4.97) and aldehyde dehydrogenase X, mitochondrial (Log\textsubscript{2}FC = 3.14). Down-regulated proteins with either the greatest fold-changes or statistical significance included: major urinary proteins 1 (Log\textsubscript{2}FC = −5.58) and 17 (Log\textsubscript{2}FC = −5.00); thyroid hormone-inducible hepatic protein (Log\textsubscript{2}FC = −4.45); and 3 beta-hydroxysteroid dehydrogenase type 5 (Log\textsubscript{2}FC = −4.28). The apoptosis maker, annexin A5 (Log\textsubscript{2}FC = 1.93) and the lipid droplet protein, perilipin-2 (PLIN2, Log\textsubscript{2}FC = 2.24), were increased in \textit{Ahr}\textsuperscript{e/e}.

\textit{Ahr}\textsuperscript{e/e} PCB126 vs. WT Vehicle was associated with 301 differentially abundant proteins (209 increased and 92 decreased)
Ahr<sup>e</sup>/<sup>e</sup> vs. WT genotype was associated with 262 differentially abundant proteins (185 increased and 77 decreased) in PCB126-exposed animals (Fig. 4D). CYP1A1 (Log<sub>2</sub>FC = -4.90) and CYP1A2 (Log<sub>2</sub>FC = -6.43) protein levels were reduced and CYP3A11 was increased (Log<sub>2</sub>FC = 2.38) in Ahr<sup>e</sup>/<sup>e</sup> PCB126 vs. WT PCB126 consistent with the gene expression data (Fig. 1). Glutathione S-transferase A1 was increased to the greatest degree (Log<sub>2</sub>FC = 5.13), while major urinary protein 1 was decreased to the greatest degree (Log<sub>2</sub>FC = -6.46). Aldehyde dehydrogenase X, mitochondrial (Log<sub>2</sub>FC = 3.06) was the most significantly increased protein and 3 beta-hydroxysteroid dehydrogenase type 5 (Log<sub>2</sub>FC = -4.59) was the most significantly decreased protein. Annexin A5 (Log<sub>2</sub>FC = 2.10) and perilipin-2 (Log<sub>2</sub>FC = 1.97) were increased while thyroid hormone-inducible hepatic protein (Log<sub>2</sub>FC = -2.67) was decreased.

In Ahr<sup>e</sup>/<sup>e</sup> mice, PCB126 exposure (vs. vehicle control) was not associated with any differentially abundant proteins (Fig. 4E).

### Table of P-values

| Outcome                  | Genotype   | PCB | Interaction | WT (Vehicle vs. PCB126) | Ahr<sup>e</sup> (Vehicle vs. PCB126) | Vehicle (WT vs. Ahr<sup>e</sup>) | PCB126 (WT vs. Ahr<sup>e</sup>) |
|--------------------------|------------|-----|-------------|-------------------------|-------------------------------------|----------------------------------|----------------------------------|
| Change in BW (%)         | 0.26       | 0.01| 0.13        | 0.75                    | 0.01                                | 0.99                             | 0.25                             |
| Body fat (%)             | <0.01      | 0.73| 0.68        | >0.99                   | 0.95                                | <0.01                            | 0.01                             |
| Lean body mass (%)       | <0.01      | 0.78| 0.64        | 0.95                    | 1.00                                | <0.01                            | 0.01                             |
| Glucose                  | <0.01      | 0.56| 0.28        | 0.65                    | 0.98                                | 0.02                             | <0.01                            |
| Insulin                  | 0.01       | 0.64| 0.41        | 1.00                    | 0.79                                | 0.56                             | 0.08                             |
| AUC                      | 0.16       | <0.01| 0.45        | 0.23                    | >0.99                               | <0.01                            | <0.01                            |
| Cholesterol              | <0.01      | 0.66| 0.31        | 0.73                    | 0.98                                | <0.01                            | <0.01                            |
| HDL-c                    | <0.01      | 0.60| 0.20        | 0.55                    | 0.95                                | <0.01                            | <0.01                            |
| Triglycerides            | 0.01       | 0.65| 0.96        | 0.99                    | 0.98                                | 0.16                             | 0.20                             |

Figure 2 Metabolic phenotyping. Body composition was determined by (A) % change in final total body weight relative to the initial total body weight; and using dual energy X-ray absorptiometry scan to obtain measurements for (B) % body fat; and (C) % lean body mass. (D) Fasting plasma glucose levels was determined by Piccolo Xpress chemical analyzer; and (E) Plasma insulin level was measured by Luminex® 100 system. A glucose tolerance test (GTT) was performed and (F) curves for GTT were plotted; in addition, (G) the area under curve (AUC) was determined. Circulating lipids, namely, fasting plasma (H) total cholesterol; (I) HDL cholesterol; and (J) triglycerides were measured using Piccolo Xpress chemical analyzer. Values are mean ± SD. A complete list of P-values (determined by two-way ANOVA with Tukey’s post-test) is provided in the accompanying table. In the figure panels, statistical significance is denoted by: a = genotype effect; b = PCB effect; c = interaction effect; d = WT Vehicle vs. WT PCB126; e = Ahr<sup>e</sup>/<sup>e</sup> Vehicle vs. Ahr<sup>e</sup>/<sup>e</sup> PCB126; f = WT Vehicle vs. Ahr<sup>e</sup>/<sup>e</sup> Vehicle; g = WT PCB126 vs. Ahr<sup>e</sup>/<sup>e</sup> PCB126. Ahr, aryl hydrocarbon receptor; PCB, polychlorinated biphenyl; WT, wild type.
Primary proteomics results are summarized in Fig. 4F. A Venn diagram for the three comparisons relative to WT Vehicle is provided as Fig. 4G. Only four proteins were shared between all experimental groups. These included: CYP1A2, CYP2C50 isoform 2, fatty acid-binding protein 5, and acyl-coenzyme A thioesterase 1 (Table S1).

### 3.5.2. Secondary proteomics analyses

Secondary proteomics data analyses were performed in MetaCore. These analyses included: enrichment by protein function, pathway enrichment by gene ontology processes and interactions by protein function.

#### 3.5.2.1. Enrichment by protein function

EPF analysis was performed to evaluate the protein classes most impacted by genotype or PCB126 exposure (Table 1). ‘Enzymes’ were the most enriched protein class (z-score range 6.42–17.84) while the ‘other’ protein class was enriched less than expected (z-score range /C03.31 to /C09.24) for the three main comparisons (e.g., WT Vehicle vs. PCB126; e = Ahr−/− Vehicle vs. Ahr−/− PCB126; f = WT Vehicle vs. Ahr−/− Vehicle; g = WT PCB126 vs. Ahr−/− PCB126. AHR, aryl hydrocarbon receptor; CD36, cluster of differentiation 36; PCB, polychlorinated biphenyl; PNPLA3, patatin-like phospholipase domain-containing protein 3; WT, wild type.

#### 3.5.2.2. Enrichment by gene ontology processes

Enrichment by GO processes was performed. For the WT (Vehicle vs. PCB126) comparison, 240 significant processes were identified (not shown). For Vehicle (WT vs. Ahr−/−) and PCB126 (WT vs. Ahr−/−), 1663 and 1947 significant processes were identified, respectively (not shown). Because there were no differentially abundant proteins detected for Ahr−/− (Vehicle vs. PCB126), there were no GO processes enriched.
These results are not surprising, given the large number of xenobiotics and organic acids; as well as the generation of energy, reduction and other reactions impacting the metabolism of lipids, enzymes involved in nitrogen metabolism, and the down-regulated 3 beta-hydroxysteroid dehydrogenase type 5. The top twenty significant over-connected objects by interactions by protein function analysis were provided in Fig. 6 and Supporting Information Fig. S4 for each intergroup comparison. Although they did not rank in the top 20 IPFs, AHR and PXR were included in Fig. 6 for internal validation. There were no significant interactions by protein function for Ahr−/− (Vehicle vs. PCB126) because there were no differentially abundant proteins for this comparison. A summarized table of the differentially expressed proteins across experimental groups is given in Table S1. This could help explain the decreased fasting blood glucose observed in these mice. Other top GO processes involved steroid metabolism, response to hormones and antibiotic biosynthesis. Ahr−/− was associated with significant alterations proteins impacting steroid synthesis, including: the upregulated 17-beta-hydroxysteroid dehydrogenase type 6 and type 13 (isofoms 1 and 2); and the down-regulated 3 beta-hydroxysteroid dehydrogenase type 5. The "dibenzo-p-dioxin metabolic process" was significantly associated with the WT (Vehicle vs. PCB126) and the PCB126 (WT vs. Ahr−/−) comparisons but not Vehicle (WT vs. Ahr−/−) (Table S1).

### Table 1: Enrichment by protein function analysis (z-score).

| Protein class | WT (Vehicle vs. PCB126) | Vehicle (WT vs. Ahr−/−) | PCB126 (WT vs. Ahr−/−) |
|---------------|-------------------------|-------------------------|-------------------------|
| Ligands       | 1.87                    | 2.60                    |                          |
| Phosphatases  | 0.90                    | −0.05                   |                          |
| Proteases     | −0.67                   | −1.05                   |                          |
| Kinases       | −0.90                   | −0.40                   |                          |
| Transcription factors | −2.91                   | −2.57                   |                          |
| Receptors     | 6.42                    | 17.84                   | 15.52                   |
| Enzymes       | −3.31                   | −9.24                   | −8.07                   |

For a given protein class, a positive z-score indicates that more proteins in that class were altered more than expected. Likewise, a negative z-score means that fewer proteins in the class were altered than expected.

Figure 4 Changes in the hepatic proteome. Alterations in hepatic proteins were demonstrated by volcano plots showing log2-transformed changes in protein abundance (vs. control) on the y-axis with log10-transformed P values on the x-axis with comparisons as follows: (A) WT Vehicle vs. WT PCB126; (B) WT Vehicle vs. Ahr−/− Vehicle; (C) WT Vehicle vs. Ahr−/− PCB126; (D) WT PCB126 vs. Ahr−/− PCB126; and (E) Ahr−/− Vehicle vs. Ahr−/− PCB126. Red denotes significantly up-regulated proteins and blue denotes significantly down-regulated proteins meeting the pre-defined fold-change (≥2-fold increase or decrease in abundance) and FDR (≤0.2) thresholds. The proteins in green met the threshold for statistical significance, but they did not meet the fold-change criterion. (F) A summarized table of the differentially expressed proteins across experimental groups. (G) Venn diagram of the differentially expressed proteins in each experimental group relative to the WT Vehicle group.
comparisons involving the Ahr−/− mice (e.g., Vehicle (WT vs. Ahr−/−) and PCB126 (WT vs. Ahr−/−) were more similar to each other than to WT (Vehicle vs. PCB126). Seven objects were common to all three comparisons including: the transcription factors, AHR, zinc finger protein 125 (ZFP125), liver X receptor α, peroxisome proliferator-activated receptor α (PPARα) and hepatocyte nuclear factor α; the insulin receptor; and miR-132-5p. The shared transcription factors possibly contributed to the observed alterations in lipid and xenobiotic metabolism. While not the top object by z-score, it was nonetheless reassuring that AHR was detected by IPF analysis.

The objects, PXR and the glucocorticoid receptor (GCR), were over-connected only for the comparisons involving Ahr−/− mice [e.g., Vehicle (WT vs. Ahr−/−) and PCB126 (WT vs. Ahr−/−)]. The PXR IPF data are consistent with the Cyp3a11 gene expression and protein abundance data presented in Fig. 1 and Table S1. Thus, the AHR and PXR IPF data were validated by internal controls. MiR-132-5p was the top object (by z-score) enriched by PCB126 in wild type mice (vs. vehicle control).

“Steroid metabolic processes” and “response to hormone” were among the top enriched GO processes (Fig. 5). Indeed, GCR and insulin receptor were among the top objects identified by IPF analyses involving the Ahr−/− mice. Thus, GCR expression and activity were determined by measuring the mRNA expression levels of Gcr and its target genes, tyrosine aminotransferase (Tat), serum/glucocorticoid regulated kinase (Sgk1) and nuclear factor kappa-inhibitor alpha (Nfkbia) by RT-PCR (Fig. 7).

| Pathway                              | Log2(Fold Change) |
|--------------------------------------|-------------------|
| Fatty acid metabolic process         | 6.60              |
| Long-chain fatty acid metabolic process | 6.58              |
| Epoxygenase P450 pathway              | 6.28              |
| Cofactor metabolic process           | 6.01              |
| Cellular lipid metabolic process      | 5.87              |
| Small molecule metabolic process      | 5.74              |
| Monocarboxylic acid metabolic process | 5.60              |
| Arachidonic acid metabolic process   | 5.26              |
| Lipid metabolic process               | 5.22              |
| Omega-hydroxylase P450 pathway       | 5.18              |
| Dibenzo-p-dioxin metabolic process   | 4.96              |
| Hydrogen peroxide biosynthetic process | 4.73              |
| Unsaturated fatty acid metabolic process | 4.64              |
| Carboxylic acid metabolic process    | 4.55              |
| Antibiotic biosynthetic process      | 4.51              |
| Icosanoid metabolic process          | 4.50              |
| Oxoacid metabolic process            | 4.37              |
| Xenobiotic metabolic process         | 4.34              |
| Cellular catabolic process           | 4.32              |
| Organic acid metabolic process       | 4.32              |
| Oxidation-reduction process          | 3.11              |
| Generation of precursor metabolites and energy | 1.73          |
| Monocarboxylic acid biosynthetic process | 2.49          |
| Organic acid biosynthetic process    | 2.07              |
| Electron transport chain             | 2.51              |
| Carboxylic acid biosynthetic process | 2.08              |
| Metabolic process                    | 3.22              |
| Drug metabolic process               | 2.72              |
| Catabolic process                    | 3.98              |
| Energy derivation by oxidation of organic compounds | 1.52   |
| Small molecule biosynthetic process  | 2.67              |
| Lipid biosynthetic process           | 2.06              |
| Small molecule catabolic process     | 2.00              |
| Steroid metabolic process            | 1.97              |
| Response to hormone                  | 1.97              |

Fig. 5 Pathway enrichment by Gene Ontology (GO) processes. Heatmap showing selected top enriched GO processes by P-value.
PCB126 exposure but not Ahr<sup>−/−</sup> genotype. However, GCR activity was increased in Ahr<sup>−/−</sup> mice, because the mRNA expression of Tat, Sgk1, and Nfkbia were increased; while PCB126 upregulated only Nfkbia (Fig. 7A–D). These data demonstrate varying degrees of GCR activation in Ahr<sup>−/−</sup> mice and with PCB126-induced ligand-activation of AHR.

PPARα and hypoxia inducible factor-1α (HIF1α) which heterodimerizes with the aryl hydrocarbon receptor nuclear translocator have been shown to regulate the expression of perilipin-2<sup>2,3,33</sup>. IPF showed over-connected interactions with PPARα and AHR, and perilipin-2 (PLIN2) protein was more abundant in Ahr<sup>−/−</sup> mice relative to WT controls. Hepatic Plin2 mRNA expression was measured by RT-PCR (Fig. 7E). Plin2 expression was altered according to genotype and was significantly higher in PCB126-exposed Ahr<sup>−/−</sup> mice relative to PCB126-exposed WT mice. There was a trend towards higher Plin2 expression in vehicle-administered Ahr<sup>−/−</sup> mice relative to vehicle-administered WT mice (P = 0.08, Fig. 7E). Thus, the perilipin-2 data are consistent at the mRNA and protein levels.

MicroRNAs are small non-coding RNAs that maintain cellular homeostasis and potentially modulate responses to environmental exposures. IPF determined enrichment in a total of nine miRs associated with PCB126 (<i>n</i> = 7), Ahr<sup>−/−</sup> (<i>n</i> = 4), or the combination of PCB126 and Ahr<sup>−/−</sup> (<i>n</i> = 3) (Supporting Information Table S2). Therefore, RT-PCR was performed to assess the hepatic expression of these nine miRs across the four

---

**Figure 6**  Interactions by protein function. Heatmap of top interactions by protein function by z-score.
groups. The expression of miR-132-5p, miR-222-3p, and miR-544-3p could not reproducibly be detected across most samples. While differential miR expression was observed for the remaining miRs, the pattern was different than predicted by IFP. Ahr⁻/⁻ was associated with significantly up-regulated miR-142-3p, miR-221-3p, miR-223-3p, and miR-150-5p and significantly down-regulated miR-192-3p. PCB126 exposure was not associated with these miRs. However, an interaction between exposure and genotype significantly decreased miR-122-5p expression in Ahr⁻/⁻ but not WT mice (Fig. S5).

4. Discussion

PCB126 activated canonical AHR signaling in WT, but not Ahr⁻/⁻, as demonstrated by increased Cyp1a1 and Cyp1a2 mRNA expression and protein abundance only in WT mice. The WT mice results confirm our previously reported findings. The 2-way ANOVA analysis, which included all four experimental groups, demonstrated several significant PCB126 effects. These included: decreased body weight gain (%); improved glucose tolerance (AUC); and increased hepatic triglycerides with altered hepatokine expression. However, on post-hoc intergroup comparison testing, PCB126 was not associated with any significant differences in the metabolic or liver phenotype of the WT mice (Fig. S5). The observed hepatic steatosis was associated with liver injury and cell death (e.g., elevated AST, ALT and annexin A5) and markedly increased mRNA expression of the hepatokine, Fgf21. Despite increased steatosis, liver weight/body weight ratio was decreased in the knockouts. Several circulating pro-inflammatory cytokines, most notably IL-6, were paradoxically decreased. Not surprisingly, the Ahr⁻/⁻ mice had 340 differentially abundant proteins to fewer than fifty. Importantly, several key consistencies were noted across studies. These include similar degrees of CYP1A1 and CYP1A2 protein up-regulation as well as the enrichment of similar metabolic pathways by PCB126. Regardless, the potential dose-responsiveness of the liver proteome to dioxin-like molecules, including PCB126, warrants future investigation. The impact of whole-body Ahr ablation was much more striking than the effects of the ligand-activation of this receptor by PCB126. Ahr⁻/⁻ mice exhibited severe metabolic disruption. Adiposity (%) and circulating leptin levels were increased, while glucose tolerance was decreased (despite decreased fasting glucose and insulin). Multiple blood and liver lipid species were increased accordingly. The observed hepatic steatosis was associated with elevated serum triglycerides with altered hepatokine expression. However, on post-hoc intergroup comparison testing, PCB126 was not associated with any significant differences in the metabolic or liver phenotype of the WT mice (vs. vehicle control). However, eight hepatic proteins were differentially abundant in WT PCB126 vs. WT Vehicle groups. All eight of these were AHR-dependent, as PCB126 exposure was associated with zero differentially abundant proteins in the knockouts (Ahr⁻/⁻ PCB126 vs. Ahr⁻/⁻ Vehicle). In a previous publication, we reported that PCB126 exposures were associated with 396 differentially abundant hepatic proteins¹⁶. The variable proteomic results across studies may potentially be explained by different: (i) statistical methods; (ii) strains of mice (Taconic vs. Jackson); (iii) diets (control vs. high fat); and (iv) exposure durations (2 vs. 12 weeks). Most notably, the present study utilized much more stringent criteria to identify significantly differentially abundant proteins (e.g., FDR < 0.05 vs. FDR < 0.2; and −1 < log₂FC < 1 vs. −0.5 < log₂FC < 0.5). Filtering the prior study’s results by the new more stringent FC criteria alone reduces the number of differentially abundant proteins to fewer than fifty. Importantly, several key consistencies were noted across studies. These include similar degrees of CYP1A1 and CYP1A2 protein up-regulation as well as the enrichment of similar metabolic pathways by PCB126. Regardless, the potential dose-responsiveness of the liver proteome to dioxin-like molecules, including PCB126, warrants future investigation.
in WT mice) with a trend towards a significant genotype-PCB126 interaction ($P = 0.09$). It was also somewhat surprising that PCB126 did not increase hepatic steatosis in WT mice vs. vehicle control. We recently reported on PCB126-induced NAFLD using a similar exposure protocol$^{19,20}$, albeit in Jackson instead of Taconic mice. While several other studies also reported PCB126-induced hepatic steatosis, these used higher doses and sometimes different routes of administration, sexes, strains, species or durations$^{13,19,34,35}$.

Secondary MetaCore analyses of the primary proteomics data were performed to characterize the principal actions of Ahr genotype or AHR ligand-activation by PCB126 in liver. Given the phenotyping data, it was not surprising that EPF analysis determined enzymes to be the major protein class effect by either activation or ablation of the Ahr, with the strongest effects occurring in the knockouts. By GO process analysis, these enzymes were chiefly involved in metabolism of lipids/steroids, xenobiotics and organic acids as well as the generation of energy. Amongst these processes, the largest number were involved with lipid metabolism. Other enriched processes included antibiotic synthesis and response to hormone.

The IPF data suggested that some of the observed effects of aryl hydrocarbon receptor ligand-activation and/or deletion may have been mediated by over-connected interactions with transcription factors previously implicated in metabolism and NAFLD. These included nuclear receptors (e.g., GCR, liver X receptor $\alpha$, PPAR$\alpha$), thyroid receptor $\beta$, hepatocyte nuclear factor $\alpha$, and PXR) and other transcription factors (ZFP125, sterol regulatory element-binding transcription factor 1, and cyclic-AMP-responsive-element-binding protein H, etc.)$^{36-38}$. As identified, altered expression of selected PXR, PPAR$\alpha$, or GCR targets was documented at the mRNA and/or protein levels in this study. The PPAR$\alpha$/aryl hydrocarbon receptor nuclear translocator target, periplin-2, was up-regulated in Ahr$^{-/-}$ mice. Previous studies have shown that PCB126 decreased expression of PPAR$\alpha$ and/or its target genes$^{19,35,39}$, and PLIN2 has previously been positively associated with NAFLD severity$^{40}$. Ahr$^{-/-}$ increased PLIN2 expression, possibly contributing to the observed NAFLD phenotype. This effect may have been indirectly mediated, occurring via over-connected interactions with the transcription factors regulating PLIN2 expression, including PPAR$\alpha$. While AHR-PPAR$\alpha$ interactions are well-documented in constitutively active and Ahr null mice$^{10,41}$, the putative interaction between the AHR and ZFP125 appears novel. ZFP125 is a FOXO1-inducible hepatic transcriptional repressor that caused NAFLD by decreasing hepatocyte lipid secretion to cause liver steatosis. It regulates several differentially abundant proteins which were associated with ligand-activation of AHR by PCB126 and/or Ahr$^{-/-}$ including fatty acid-binding protein 5 and apolipoprotein A4$^{38}$.

It was previously demonstrated that the GCR potentiates rat CYP1A1 induction by polycyclic aromatic hydrocarbons via direct interaction of the GCR with glucocorticoid response elements located in intron 1 of Cyp1a1$^{42}$. Here, we show that either ligand-activation or ablation of the aryl hydrocarbon receptor variably increased GCR target gene expression, suggesting that the AHR may also modulate GCR activity. This finding may have implications for human health. For example, some AHR ligands have demonstrated anti-inflammatory actions in fatty liver disease models$^{43,44}$. Perhaps these AHR-ligands could increase glucocorticoid receptor signaling to decrease liver inflammation. However, in the present study, the Ahr$^{-/-}$ genotype was associated with reduced circulating pro-inflammatory cytokines, while PCB126 exposure did not alter these mediators. More data are clearly required to better understand interactions between the AHR and the transcription factors elucidated by the enrichment by IPF analysis. However, cross-regulation of Ahr and Gcr mRNA expression has been reported$^{45}$.

To our knowledge, miRs have not previously been associated with Ahr ablation, but they have been associated with PCB exposures. Here IPF elucidated enriched interactions between nine miRs. The majority of these miRs were confirmed to be differentially expressed by RT-PCR, although not usually in the same pattern predicted by the IPF analysis. While the discrepancy between IPF and RT-PCR results could be explained if one set of results was incorrect, alternative explanations may exist (e.g., small miR-quenching RNAs). Interestingly three of the miRs with significantly different liver expression by RT-PCR (e.g., miR-122-5p, miR-221-3p, and miR-192-3p) were also associated with liver disease in an abstract evaluating circulating miRs in a human cohort with high residential PCB exposures$^{46}$. The initial miR findings from the present manuscript require confirmation and additional investigation. Nonetheless, the potential role of miRs in AHR-regulated liver homeostasis is an unanticipated, yet interesting, future research direction.

Major urinary proteins (MUPs) 1 and/or 17 were among the most highly down-regulated proteins identified in the Ahr$^{-/-}$ mice. As such, they warrant discussion. MUPs are unique members of the lipocalin superfamily that mediate both chemical and metabolic signaling$^{47}$. MUPs bind and stabilize pheromones to regulate their transport and release into the air from urine. MUPs also regulate carbohydrate and lipid metabolism. Recombinant MUP1 has anti-diabetic actions. It suppressed hepatic gluconeogenesis while stimulating mitochondrial biogenesis in skeletal muscle to increase energy expenditure$^{47}$. Little is known about the AHR and MUPs, but 2,3,7,8-tetrachlorodibenzo-p-dioxin repressed the signal transducer and activator of transcription 5b (STAT5b) target gene, Mup2, in an AHR-dependent manner$^{48}$. These data suggest the possibility that pheromones could be disrupted by environmental chemicals interacting with the AHR. Perhaps the effects of pheromones on chemical signaling and metabolism warrant additional investigation in the environmental health sciences (pheromone disruption).

While genetic knockout approaches are generally strong, the approach taken in the manuscript may be limited by the differential performance of the various strains of Ahr$^{-/-}$ mice on liver and metabolic endpoints in the published literature. While these differences could be related to genetic differences caused by the specific approaches used to ablate the Ahr, they could also be due to other factors including genetic background, diet, age, sex, microbiome, etc. To clarify, the Ahr$^{-/-}$ mice (C57Bl/6 background) utilized in this study were obtained from Taconic. This strain was derived by CXR Biosciences from a humanized AHR Mouse line through a Cre-mediated deletion of the human AHR sequence, and it is a whole-body knockout. Mouse exon 3 is deleted in the knockout, resulting in an out of frame splicing of exons 2 to exon 4. Strain specific-differences have been reported between the whole-body Ahr$^{-/-}$ generated by the Bradfield and Gonzalez groups (both of which are on similar genetic backgrounds—mixed C57Bl/6 and 129SV)$^{13,21}$. While these strains have significantly smaller livers, only the Bradfield strain was reported to have increased early-life hepatic steatosis which improved over time. In a 14 week feeding study, male Bradfield mice were protected from high fat diet (HFD)-induced obesity and
displayed improved insulin signaling. Liver-specific knockouts generated using Bradfield’s conditional Ahr−/− model (on a 129SV background) had increased HFD-induced hepatic steatosis (vs. WT controls)25,26. Liver cell-specific knockouts have likewise been generated from this conditional model, demonstrating that Ahr ablation in stellate cells was associated with increased CCl4-induced fibrosis25. However, these models were criticized due to the lower-affinity AHR present in 129SV mice, prompting the recent development of a series of new conditional knockouts using CRISPR-Cas927. Likewise, a novel tamoxifen-inducible and hepatocyte-specific Ahr−/− mouse model, had significantly reduced adiposity attributed to increased production of the thermogenic hepatokine, FGF2128. Based on this literature, it is possible that our results might not be generalizable to other Ahr−/− strains. Future studies should address several related limitations of the present manuscript. For example, tissue-specific (e.g., liver, intestine, etc.) Ahr knockouts are required to dissect the direct effects of hepatic AHR from the systemic effects of extrahepatic AHR indirectly impacting the liver proteome. These studies could also include integrated multi-‘omics analyses (e.g., transcriptomics, metagenomics, phosphoproteomics, etc.) to better clarify AHR’s role in liver health and disease.

Overall, the metabolic phenotyping and proteomic data converged on Ahr’s critical role in the coordination and maintenance of systemic energy and lipid homeostasis. Perturbing this system influenced the development of obesity-associated diseases. For example, Ahr−/− mice had increased adiposity, hepatic steatosis and glucose intolerance with paradoxically decreased blood lipids. Mechanistically, Ahr mainly targeted liver metabolism (e.g., lipids, xenobiotics, organic acids, etc.), bioenergetics and endocrine function (e.g., production of hepatokines, steroids and pheromone binding proteins). Some of these effects may have been indirectly mediated by the interacting miRs or the transcription factors identified by MetaCore. While the proteome was impacted to a greater degree by Ahr−/− than by PCB126-induced AHR activation, the top GO processes identified were similar. Interestingly, the PCB126-associated liver proteome was entirely Ahr-dependent. Based on the literature, it is possible that some results could be dependent on the specific strain of Ahr−/− mouse utilized. Regardless, the Ahr and its ligands warrant more research in metabolic health and disease.

Acknowledgments

This research was supported, in part, by the National Institute of Environmental Health Sciences (R35ES028373, R01ES032189, T32ES011564, P42ES023716, P30ES030283, F31ES028982 and R21ES031510, USA); the National Institute of General Medical Sciences (P20GM113226, USA); the National Institute on Alcohol Abuse and Alcoholism (P50AA024337 and 1F32AA027950, USA); the Kentucky Council on Postsecondary Education (PON2 415 1900002934, USA); and the Wendell Cherry Endowed Chair. Dr. Jin would like to acknowledge the contributions of several members of his doctoral dissertation committee who were not included as coauthors (e.g., Drs. Daniel J. Conklin, Sri Prakash Mokshagundam, Joshua L. Hood, and Jonathan H. Freedman). This individuals helped to shape the direction of this research. Daniel Wilkey, Dr. Keith Cameron Falkner, Sydney E. Smith, Erica F. Daly and Steve Mahanes are also acknowledged.

Author contributions

The authors contributed as follows: Jian Jin (investigation, visualization, writing — original draft); Banrida Wahlang (investigation, formal analysis, writing — review & editing); Monika Thapa (investigation); Kimberly Z. Head (investigation); Josiah E. Hardesty (investigation, methodology, data curation); Sudhri Srivastava (formal analysis); Michael L. Merchant (methodology, resources, supervision); Shesh N. Rai (formal analysis, resources, supervision); Russell A. Prough (conceptualization; writing — review & editing); and Matthew C. Cave (conceptualization, funding acquisition, project administration, resources, supervision, writing, review & editing).

Conflicts of interest

The authors have no conflicts of interest relevant to this research. All authors have approved the final version of the manuscript.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2021.10.014.

References

1. Tapper EB, Parikh ND. Mortality due to cirrhosis and liver cancer in the United States, 1999-2016: observational study. BMJ 2018;362:k2817.
2. Wahlang B, Beier JJ, Clair HB, Bellis-Jones HJ, Falkner KC, McClain CJ, et al. Toxicant-associated steatohepatitis. Toxicol Pathol 2013;41:343–60.
3. Wahlang B, Jin J, Beier JJ, Hardesty JE, Daly EF, Schnegelberger RD, et al. Mechanisms of environmental contributions to fatty liver disease. Curr Environ Health Rep 2019;6:80–94.
4. Foulds CE, Trevino LS, York B, Walker CL. Endocrine-disrupting chemicals and fatty liver disease. Nat Rev Endocrinol 2017;13:445–57.
5. Heindel JJ, Blumberg B, Cave M, Machtinger R, Mantovani A, Mendez MA, et al. Metabolism disrupting chemicals and metabolic disorders. Reprod Toxicol 2017;68:3–33.
6. Allard J, Le Guillo D, Begriche K, Fromenty B. Drug-induced liver injury in obesity and nonalcoholic fatty liver disease. Adv Pharmacol 2019;85:75–107.
7. Cave M, Falkner KC, Ray M, Joshi-Barve S, Brock G, Khan R, et al. Toxicant-associated steatohepatitis in vinyl chloride workers. Hepatology 2010;51:474–81.
8. Murray IA, Patterson AD, Perdew GH. Aryl hydrocarbon receptor ligands in cancer: friend and foe. Nat Rev Cancer 2014;14:801–14.
9. Esser C, Rannug A. The aryl hydrocarbon receptor in barrier organ physiology, immunology, and toxicology. Pharmacol Rev 2015;67:259–79.
10. Lee JH, Wada T, Febrero M, He J, Matsubara T, Lee MJ, et al. A novel role for the dioxin receptor in fatty acid metabolism and hepatic steatosis. Gastroenterology 2010;139:653–63.
11. Kim JH, Matsubara T, Lee J, Fenollar-Ferrer C, Han K, Kim D, et al. Lysosomal SLC46A3 modulates hepatic cytosolic copper homeostasis. Nat Commun 2021;12:290.
12. Jurgelewicz A, Dornbos P, Warren M, Nault R, Arkatkar A, Lin H, et al. Genetics-based approach to identify novel genes regulated by the aryl hydrocarbon receptor (AHR) in mice liver. Toxicol Sci 2021;181:285–94.
Liver proteomics and the AHR 3819

13. Yan J, Tung HC, Li S, Niu Y, Garbacz WG, Lu P, et al. Aryl hydrocarbon receptor signaling prevents activation of hepatic stellate cells and liver fibrogenesis in mice. *Gastroenterology* 2019;157:793–806.e14.

14. Perkins JT, Petretti MC, Newsome BJ, Hennig B. Polychlorinated biphenyls and links to cardiovascular disease. *Environ Sci Pollut Res Int* 2016;23:2160–72.

15. Girer NG, Carter D, Bhattachar N, Mustafa M, Denner L, Porter C, et al. Inducible loss of the aryl hydrocarbon receptor activates peroxi

16. Jin J, Wahlang B, Shi H, Hardesty JE, Falkner KC, Head KZ, et al. Dioxin-like and non-dioxin-like PCBs differentially regulate the hepatic proteome and modify diet-induced nonalcoholic fatty liver disease severity. *Med Chem Res* 2020;29:1247–63.

17. Wahlang B, Hardesty JE, Jin J, Falkner KC, Cave MC. Polychlorinated biphenyls and nonalcoholic fatty liver disease. *Curr Opin in Toxicol* 2019;14:21–8.

18. Wahlang B, Perkins JT, Petretti MC, Hoffman JB, Stromberg AJ, Hennig B. A compromised liver alters polychlorinated biphenyl-mediated toxicity. *Toxicology* 2017;380:11–22.

19. Gadupudi GS, Klaren WD, Olivier AK, Klingelhoitz AJ, Robertson LW. PCB126-induced disruption in gluconeogenesis and fatty acid oxidation precedes fatty liver in male rats. *Toxicol Sci* 2016;149:98–110.

20. Schmidt JV, Su GH, Reddy JK, Simon MC, Bradfield CA. Characterization of a murine Ahr null allele: involvement of the Ah receptor in hepatic growth and development. *Proc Natl Acad Sci U S A* 1996;93:6731–6.

21. Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, et al. Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science* 1995;268:722–6.

22. Xu CX, Wang C, Zhang ZM, Jaeger CD, Krager SL, Bottum KM, et al. Aryl hydrocarbon receptor deficiency protects mice from diet-induced adiposity and metabolic disorders through increased energy expenditure. *Int J Obes (Lond)* 2015;39:1300–9.

23. Wada T, Sunaga H, Miyata K, Shirasaki H, Uchiyama Y, Shimba S. Aryl hydrocarbon receptor plays protective roles against high fat diet (HFD)-induced hepatic steatosis and the subsequent lipotoxicity via direct transcriptional regulation of Socs3 gene expression. *J Biol Chem* 2016;291:7004–16.

24. Wallisser JA, Glover E, Pande K, Liss AL, Bradfield CA. Aryl hydrocarbon receptor-dependent liver development and hepatotoxicity are mediated by different cell types. *Proc Natl Acad Sci U S A* 2005;102:17858–63.

25. Shi H, Jan I, Hardesty JE, Falkner KC, Prough RA, Balamurugan AN, et al. Polychlorinated biphenyl exposures differentially regulate hepatic metabolism and pancreatic function: implications for nonalcoholic steatohepatitis and diabetes. *Toxicol Appl Pharmacol* 2019;363:22–33.

26. Wahlang B, Jin J, Hardesty JE, Head KZ, Shi H, Falkner KC, et al. Identifying sex differences arising from polychlorinated biphenyl exposures in toxicant-associated liver disease. *Food Chem Toxicol* 2019;129:64–76.

27. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 1959;37:911–7.

28. Wiśniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. *Nat Methods* 2006;6:589–62.

29. Keshishian H, Burgess MW, Gillette MA, Mertins P, Clausew KR, Mani DR, et al. Multiplexed, quantitative workflow for sensitive biomarker discovery in plasma yields novel candidates for early myocardial injury. *Mol Cell Proteomics* 2015;14:2375–93.

30. McDowell GS, Gaun A, Steen H. iFASP: combining isobaric mass tagging with filter-aided sample preparation. *J Proteome Res* 2013;12:3809–12.

31. Srivastava S, Merchant M, Rai A, Rai SN. Standardizing proteomics workflow for liquid chromatography-mass spectrometry: technical and statistical considerations. *J Proteomics Bioinform* 2019;12:48–55.