The essential roles of FXR in diet and age influenced metabolic changes and liver disease development: a multi-omics study

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Abstract

Background

Aging and diet are risks for metabolic diseases. Bile acid receptor farnesoid X receptor (FXR) knockout (KO) mice develop metabolic liver diseases that progress into cancer as they age, which is accelerated by Western diet (WD) intake. The current study uncovers the molecular signatures for diet and age-linked metabolic liver disease development in an FXR-dependent manner.

Methods

Wild-type (WT) and FXR KO male mice, either on a healthy control diet (CD) or a WD, were euthanized at the ages of 5-, 10-, or 15-months. Hepatic transcriptomics, liver, serum, and urine metabolomics as well as microbiota were profiled.

Results

WD intake facilitated hepatic aging in WT mice. In an FXR-dependent manner, increased inflammation and reduced oxidative phosphorylation were the primary pathways affected by WD and aging. FXR has a role in modulating inflammation and B cell-mediated humoral immunity which was enhanced by aging. Moreover, FXR dictated neuron differentiation, muscle contraction, and cytoskeleton organization in addition to metabolism. There were 654 transcripts commonly altered by diets, ages, and FXR KO, and 76 of them were differentially expressed in human hepatocellular carcinoma (HCC) and healthy livers. Urine metabolites differentiated dietary effects in both genotypes, and serum metabolites clearly separated ages irrespective of diets. Aging and FXR KO commonly affected amino acid metabolism and TCA cycle. Moreover, FXR is essential for colonization of age-related gut microbes. Integrated analyses uncovered metabolites and bacteria linked with hepatic transcripts affected by WD intake, aging, and FXR KO as well as related to HCC patient survival.

Conclusion

FXR is target to prevent diet or age-associated metabolic disease. The uncovered metabolites and microbes can be diagnostic markers for metabolic disease.

Background

Diet and aging are major risk factors for metabolic diseases including non-alcoholic steatohepatitis (NASH), which leads to the development of hepatocellular carcinoma (HCC) [1, 2]. Currently, there is no drug that can be used to treat NASH, and the outcome of HCC treatment remains unsatisfactory. Early detection and identification of treatment targets are essential to moving the field forward.
With the use of fecal transplantation, antibiotic treatment, probiotic and prebiotic interventions, as well as dietary supplementation, it is now clear that gut microbes and their metabolites contribute to metabolic disease development [3–6]. One of the established mechanisms includes that diet-associated dysbiosis accompanied by dysregulated bile acid (BA) synthesis leading to IL17A production and systemic inflammation [7–9]. Thus, diet- and aging-associated gut microbes and metabolites can be biomarkers for metabolic liver disease development.

BAs are synthesized from hepatic metabolism of dietary cholesterol and are further metabolized by bacterial enzymes to generate secondary BAs in the intestine [10]. The beneficial or toxic effects of BAs depend on their composition and concentration. The metabolic, detoxification, and immune functions of BAs are mediated through their receptors, among which FXR (farnesoid X receptor) and GPBAR1 (G protein-coupled bile acid receptor 1) have been studied extensively [10]. Because FXR regulates BA homeostasis, its knockout (KO) in mice causes BA synthesis dysregulation and leads to the development of non-alcoholic fatty liver (5-months old), which progresses into NASH (10-months old) and HCC (15-months old) even when FXR KO mice consume a healthy diet [11, 12].

Patients who have liver cirrhosis or HCC have reduced FXR [13]. Our previous study reported that Western diet (WD) intake facilitates the tumorigenesis of FXR KO mice in a sex-dependent manner [5, 14]. Moreover, WD-induced systemic inflammation is accompanied by BA receptor deactivation [7, 14]. In contrast, probiotics-prevented hepatic inflammation is associated with restoration of BA receptor-regulated signaling [6]. These findings revealed the significance of dysregulated BA, which is noted in FXR KO mice, in contributing to metabolic liver disease development.

The current study used multi-omics approaches to uncover the impact of diet, age, and FXR functional status on gut microbiota, metabolites (liver, urine, serum), and hepatic phenotypes based on transcriptomic pathways. Our novel data revealed the essential roles of FXR in combating diet and age-associated metabolic liver disease development. Additionally, the data revealed the molecular signatures within the gut-liver axis implicated in the development of non-alcoholic fatty liver disease (NAFLD) and HCC.

Methods

Specimens and animals

The specimens used were derived from mice with established phenotypes [4, 5, 14, 15]. Wild-type (WT) and FXR KO [16] male mice were specific pathogen-free with the genetic background of C57BL/6N. Mice were fed either a healthy control diet (CD, TD.140415; 5.2% fat, 12% sucrose, and 0.01% cholesterol, w/w) or a Western diet (WD, TD.140414; 21.2% fat, 34% sucrose, and 0.2% cholesterol, w/w) (Harlan Teklad, Madison, WI) since weaning (3 weeks) and euthanized at the age of 5, 10, and 15 months. Diet and water were provided ad libitum. Experiments were conducted in accordance with the NIH Guide for the Care and
Use of Laboratory Animals under protocols approved by the Institutional Animal Care and Use Committee of the University of California, Davis (Sacramento, CA).

**Untargeted metabolomic study**

Hepatic metabolites (n = 6/group) were analyzed by gas chromatography–time-of-flight mass spectrometry (GC-TOF-MS) at the West Coast Metabolomics Center (University of California, Davis). All database entries in BinBase were filtered and matched against a Mass Spectral Library of 1,200 authentic metabolites spectra with retention index and mass spectrum information or against the National Institution of Standards and Technology library.

**Quantification of serum and urine metabolites**

Serum and urine metabolites (n = 6/group) were quantified by NMR using published methods (9). Briefly, samples were filtered by Amicon Ultra-0.5 mL centrifugal filter. Avance 600 MHz NMR spectrometer (Bruker, Billerica, MA) equipped with a SampleJet was used for NMR data acquisition. The concentrations of serum and urine metabolites were normalized by log transformation [15].

**RNA sequencing and data processing**

Hepatic RNA was extracted (n = 4) using TRIzol reagent (Invitrogen, Carlsbad, CA). The liver specimens used were from the same cohort of mice used in previous publications [4, 5, 14, 15]. RNA-sequencing was performed by Novogene Co., LTD (Sacramento, CA). Raw sequence reads (FASTQ) data were mapped to the reference mouse transcriptome index (GRCm39/mm39, GENCODE release M27) and quantified with Salmon (version 1.4.0) pseudoaligner [17]. Gene-level counts were imported with tximport [18] and differential expression analysis was performed with DESeq2 (version 1.18) using the lfcShrink function [19] in R 4.03.

**Human databases**

RNA sequencing data (normalized RSEM values) for 371 liver hepatocellular carcinoma and 50 normal control samples were sourced from The Cancer Genome Atlas (TCGA) database.

**Bioinformatics**

Principal component analysis (PCA), statistical analysis, and quantitative enrichment analysis for metabolites were conducted with MetaboAnalyst 5.0 (https://www.metaboanalyst.ca). PCA of transcriptomic data was performed using R 4.2.0. Normalized read counts were used for Gene Set Enrichment Analysis (GSEA) [20]. Pathway analyses of the differentially expressed genes (DEGs) were performed using Metascape (http://metascape.org). The overall survival rate was assessed using OncoLnc (http://www.oncolnc.org/).

**Statistics**

Metabolite levels were statistically significant if the uncorrected \( p \) value < 0.05 and false discovery rate (FDR) corrected \( p \) value < 0.1. Genes with FDR corrected \( p \)-value < 0.05 and absolute fold change \( \geq 2 \) were
considered as differentially expressed. Spearman's correlation was used to assess the relationship between hepatic features (transcripts and metabolites) and non-hepatic features (serum and urine metabolites as well as gut microbiota at the genus level), and a significant correlation was defined when the Hochberg-adjusted $p$ value $< 0.05$. Data are expressed as the mean ± SD. *$p< 0.05$ was considered significantly different.

**Results**

**Hepatic phenotypes**

Hepatic phenotypes have been reported [3–5, 14], and the findings are summarized as follows. WD consumption induced steatosis in WT and FXR KO mice of all ages in a time-dependent manner. Healthy CD-fed 5-month-old FXR KO mice developed steatosis which increased in severity with age [14]. WD intake exacerbated the pathology in FXR KO mice, resulting in the development of NASH at 10 months of age [5], and liver tumors at 15 months of age [4].

**WD-altered hepatic transcripts and pathways were largely dependent on FXR function**

Principal component analysis (PCA) of hepatic transcriptomes showed that CD- and WD-fed mice formed two clusters in WT mice but not in FXR KO mice (Fig. 1A). In addition, the number of differentially expressed genes (DEGs) based on diets was much smaller in FXR KO mice (486) than in WT mice (2,250) (Fig. 1B). There were 36 transcripts consistently altered by WD in WT mice irrespective of their ages, but only 6 (Scd3, Cidec, Csad, Cyp39a1, Dntt, and 9130409I23Rik) were found in FXR KO mice (Fig. S1). Those 36 transcripts might represent the hallmarks of WD intake. The functions of those transcripts are summarized in Supplementary Table S1.

Pathway analyses were performed for the 2,250 DEGs from WT and 486 DEGs in FXR KO mice. WD increased cytokine production and inflammation but downregulated oxidative phosphorylation (OXPHOS) only in WT mice. Common pathways that changed due to WD intake in both genotypes were: downregulation of genes implicated in cholesterol biosynthesis, oxidoreductase activity, isoprenoid metabolic processes, and nucleoside bisphosphate metabolic processes (Fig. 1C, $p < 0.05$ and enrichment factor $\geq 1.5$). In FXR KO mice, WD uniquely upregulated iron binding but downregulated fatty acid metabolism genes (Fig. 1C). Together, when FXR is deactivated, many WD-regulated genes and pathways were no longer differentially found in WT mice, indicating the similarity between WD intake and FXR inactivity.

**Dietary effects on the liver, serum, and urine metabolome as well as gut microbiota in WT and FXR KO mice**

Similar to the transcriptome results, PCA of the hepatic metabolome datasets revealed that the effects of diet were more distinctive in WT than in FXR KO mice (Fig. 2A). The number of metabolite changes due to
differential dietary intake was larger in WT (67) than that in FXR KO (41) mice (Fig. 2B). The diet-altered metabolites commonly or specifically found in WT and FXR KO mice are shown in Fig. S2. Irrespective of ages, WD intake increased hepatic myo-inositol, squalene, glutamine, oxoproline, and uracil but reduced 2-hydroxybutanoic acid, 2-aminobutyric acid, and linoleic acid in WT mice. In FXR KO mice, only hepatic linoleic acid was reduced by WD intake in those 3 age groups (Fig. 2B). Quantitative enrichment analysis showed that arginine biosynthesis, propanoate metabolism, alanine, aspartate and glutamate metabolism, linoleic acid metabolism, cysteine and methionine metabolism, and glyoxylate and dicarboxylate metabolism were altered by WD for both genotypes (purple, Fig. 2C). For FXR KO mice, WD intake altered citrate cycle (succinic acid, citric acid, fumaric acid), pyruvate metabolism (fumaric acid), biosynthesis of unsaturated fatty acids (palmitic acid, linoleic acid, arachidonic acid), and glycolysis/gluconeogenesis (3-phosphoglyceric acid) (black, Fig. 2C).

In contrast to hepatic metabolites, the impact of FXR KO on serum metabolites due to differential diet intake was not obvious when all ages were considered together based on PCA (Fig. S3). However, the impact of diet on urine metabolites was obvious irrespective of ages in both genotypes suggesting the sensitivity of urine metabolites to detect dietary differences (Fig. S4).

Interestingly upon PCA of the cecal microbiota, the dietary differences were apparent for younger mice (5, and 10-months old, triangles and squares), but not in 15-months old (circles) WT mice, indicating the dietary influence on the gut microbiota was affected by feeding duration or aging (Fig. S5). In contrast, in FXR KO mice, CD and WD groups formed two distinct clusters suggesting the usefulness of microbiota to differentiate FXR status (Fig. S5).

**The significance of FXR on hepatic transcriptomic changes based on ages**

The PCA plot shown in Fig. 1A suggests that WD intake may facilitate liver aging. Specifically, CD-fed 5- and 10-month-old mice clustered distinctly from the 15-months old mice. However, in WD-fed mice, the 10- and 15-month-old groups formed a cluster. Thus, age-related changes in the hepatic transcriptome were accelerated in WD-fed mice.

The effects of age (15 vs. 5 months old) on the transcriptomes were further analyzed. PCA of hepatic transcriptomes differentiated ages in WT but not in FXR KO mice (Fig. 3A). Many more DEGs were found in WT (1,908), due to age differences, than in FXR KO (470) mice (Fig. 3B). Similar to the dietary effects, more genes involved in inflammatory response and leukocyte migration functional categories were upregulated by aging in WT than FXR KO mice (Fig. 3C). In FXR KO mice, aging uniquely upregulated the hematopoietic cell lineage and B cell receptor signaling pathways (Fig. 3C). In addition, age-downregulated OXPHOS genes were only noted in WT mice, highlighting the significance of FXR in OXPHOS.
Age-related changes of metabolites and cecal microbiota in WT and FXR KO mice

Although the number of liver metabolites altered by age in FXR KO (n = 48) was smaller than that in WT mice (n = 65), two clusters were still noted based on age (15 vs. 5) in both WT and FXR KO mice as shown in Fig. 4A-B. In both genotypes, aging elevated hepatic xylitol but reduced fructose and linoleic acid irrespective of the type of diet (Fig. 4B, Fig.S6). The top 10 metabolic pathways affected by age in each genotype are summarized in Fig. 4C. Among them, biosynthesis of unsaturated fatty acids, arginine biosynthesis, galactose metabolism, and fructose and mannose metabolism were affected by age in both genotypes (Fig. 4C). Moreover, aging decreased hepatic ethanolamine and metabolites involved in the aminoacyl-tRNA biosynthesis in WT mice revealing the significance of FXR in regulating hepatic amino acid/peptide synthesis (Fig. 4C, Fig. S6).

Serum and urine metabolites were also differentiated by age effects in both genotypes (Fig. S7, Fig. S8), but the numbers of metabolites changed due to age differences were always larger in WT mice than those in FXR KO mice (Fig. S7B, Fig. S8B). Collectively, the data suggest that FXR plays a significant role in age-linked metabolic changes. Consistent with transcriptomic changes, the age-mediated differences in the cecal microbiota at the genus level were not obvious in FXR KO mice (Fig. S9A). Taken together, the findings suggest that FXR dictates the age-associated gut microbiota community structure.

Molecular signatures alterations due to FXR deficiency

PCA revealed that FXR inactivation shifted the hepatic transcriptomes of both CD- and WD-fed mice of different ages, as revealed by the alteration in expression of 2,383 and 1,627 transcripts in CD and WD groups, respectively (Fig. 5A). In both dietary groups, common pathways that shifted due to lack of FXR are shown in Fig. 5C. Remarkably, when mice were on a WD, FXR deactivation led to enhanced chemical carcinogenesis, extracellular matrix structural constituent, etc., suggestive of hepatocarcinogenicity impacted by WD in combination with lack of FXR (Fig. 5C).

For hepatic metabolites, FXR KO mice had reduced sugars (e.g., melibiose and glucoheptulose) irrespective of diets or ages (Fig. 6B, Fig. S10). Additionally, lack of FXR increased hepatic fumaric acid, malic acid, and parabanic acid (Fig. S10A). There was a combined effect of diet, age, and FXR KO in which more changes were noted in WD-fed FXR KO mice at the age of 15 months when the mice had liver tumors [4]. For example, hepatic orotic acid, 3-phosphoglycerate, levoglucosan, alanine-alanine, 3,6-anhydrous-D-galactose, glucose, galactitol, xylitol, erythritol, and xanthosine were accumulated in 15-month-old WD-fed FXR KO mice, which were not seen in WT mice of the same age on the WD (Fig. S10B; highlighted in red). FXR KO altered amino acid and propanoate metabolism (purple, Fig. 6C). Pyrimidine metabolism, tyrosine metabolism, and pyruvate metabolism as well as glycosylphosphatidylinositol-anchor biosynthesis and citrate cycle pathways affected by lacking FXR were uniquely found in WD-fed mice (Fig. 6C; down, marked in black). These changes may reflect the cancer phenotype developed by FXR deactivation and WD intake.
Further analysis revealed that changes in FXR KO linked serum metabolite were only apparent in the CD group revealing the overlapping effects of WD intake and FXR KO (Fig. S11A). Serum 2-hydroxybutyrate increased in FXR KO mice at all ages irrespective of diets (purple, Fig. S11B, Fig. S12). In contrast to serum metabolites, PCA plots showed that urine metabolites clustered into two distinct groups based on the mouse genotypes in both CD and WD-fed mice (Fig. S13). FXR KO increased urine 2-hydroxyvalerate and acetoacetate but reduced taurine, creatinine, and trimethylamine (TMA) irrespective of diets (purple, Fig. S13, Fig. S14). In addition to urine metabolites, the cecal microbiota distinguished WT and FXR KO (Fig. S15). Notably, cecal *Bacteroides* were increased by FXR deactivation regardless of the provided diet type (purple, Fig. S15B-C).

**Common changes due to WD intake, aging, and FXR KO**

The expression levels of a total of 654 transcripts were commonly altered due to differential diet intake, ages, and FXR functionality (Fig. 7A). Among them, 76 transcripts have altered expression levels in human HCC specimens compared with healthy livers using the TCGA database (Fig. 7A). Pathway analysis of those 76 transcripts revealed their roles in cell division, mitotic spindle, and cancer development (Fig. 7C). Furthermore, 18 out of those 76 transcripts were upregulated in HCC and their expression levels were positively associated with poor overall survival in HCC patients (Table 1). Those 18 genes are *PLEKHH1, TTC39A, ATP6V0D2, CENPE, KIF20A, ASPM, CKAP2L, HMMR, ECT2, TOP2A, KIF18B, TPX2, NUF2, TRIM59, FRZB, E2F8, TREM2, and MTHFD1L*. Additionally, 7 downregulated *PCK1, TSC22D3, SLC22A7, CYP2U1, SARDH, TTC36, and APOC1* were related to a worse overall survival rate in HCC patients suggesting their tumor suppressive effects. The known functions of these transcripts are summarized in Table 1. These findings revealed the human liver cancer relevance of our findings, produced in diet and aging mouse models, and most of which did not have liver cancer.
Table 1
Transcripts that are commonly changed by WD, aging, and FXR KO are associated with the overall survival rate in HCC patients (p value < 0.05). The “high” and “low” expression was defined as the upper and lower quartiles of expression for each gene (n = 108, 50% as a cutoff for both lower and upper quartiles)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Full Name</th>
<th>HCC vs. Normal</th>
<th>Association</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLEKHH1</td>
<td>pleckstrin homology, MyTH4 and FERM domain containing H1</td>
<td>Up</td>
<td>Unfavored</td>
<td>Unknown</td>
</tr>
<tr>
<td>TTC39A</td>
<td>tetratricopeptide repeat domain 39A</td>
<td>Up</td>
<td>Unfavored</td>
<td>Unknown</td>
</tr>
<tr>
<td>ATP6V0D2</td>
<td>ATPase H+ transporting V0 subunit d2</td>
<td>Up</td>
<td>Unfavored</td>
<td>It is a subunit of the integral membrane V0 complex of vacuolar ATPase which is responsible for acidifying a variety of intracellular compartments in eukaryotic cells, thus providing most of the energy required for transport processes in the vacuolar system. Regulator of osteoclast fusion and bone formation</td>
</tr>
<tr>
<td>CENPE</td>
<td>centrosome-associated protein E</td>
<td>Up</td>
<td>Unfavored</td>
<td>Microtubule motor activity</td>
</tr>
<tr>
<td>KIF20A</td>
<td>kinesin family member 20A</td>
<td>Up</td>
<td>Unfavored</td>
<td>Microtubule motor activity</td>
</tr>
<tr>
<td>ASPM</td>
<td>abnormal spindle microtubule assembly</td>
<td>Up</td>
<td>Unfavored</td>
<td>Involved in mitotic spindle regulation and coordination of mitotic processes (35)</td>
</tr>
<tr>
<td>CKAP2L</td>
<td>cytoskeleton associated protein 2 like</td>
<td>Up</td>
<td>Unfavored</td>
<td>Microtubule-associated protein required for mitotic spindle formation and cell-cycle progression in neural progenitor cells</td>
</tr>
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Superscript is the information of references related to human HCC.
<table>
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<tr>
<td></td>
<td></td>
<td>WD vs. CD</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>15 vs. 5</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>FXR KO vs. WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMMR</td>
<td>hyaluronan mediated motility receptor</td>
<td>Up</td>
<td>Unfavored</td>
<td>Involved in cell motility (29)</td>
</tr>
<tr>
<td>ECT2</td>
<td>epithelial cell transforming 2</td>
<td>Up</td>
<td>Unfavored</td>
<td>A guanine nucleotide exchange factor and transforming protein that is related to Rho-specific exchange factors and yeast cell cycle regulators (33)</td>
</tr>
<tr>
<td>TOP2A</td>
<td>DNA topoisomerase II alpha</td>
<td>Up</td>
<td>Unfavored</td>
<td>A DNA topoisomerase, an enzyme that controls and alters the topologic states of DNA during transcription (32)</td>
</tr>
<tr>
<td>KIF18B</td>
<td>kinesin family member 18B</td>
<td>Up</td>
<td>Unfavored</td>
<td>Mitotic cell cycle; Microtubule motor activity; Protein binding; ATP binding; Kinesin binding (31)</td>
</tr>
<tr>
<td>TPX2</td>
<td>TPX2 microtubule nucleation factor</td>
<td>Up</td>
<td>Unfavored</td>
<td>Apoptotic process; Mitotic nuclear division; Cell proliferation; Activation of protein kinase activity; Regulation of Mitotic spindle organization (27)</td>
</tr>
<tr>
<td>NUF2</td>
<td>NUF2 component of NDC80 kinetochore complex</td>
<td>Up</td>
<td>Unfavored</td>
<td>A component of a conserved protein complex associated with the centromere (30)</td>
</tr>
<tr>
<td>TRIM59</td>
<td>tripartite motif containing 59</td>
<td>Up</td>
<td>Unfavored</td>
<td>Negative regulation of I-kappaB kinase/NF-kappaB signaling (26)</td>
</tr>
<tr>
<td>FRZB</td>
<td>frizzled related protein</td>
<td>Up</td>
<td>Unfavored</td>
<td>Negative regulation of cell proliferation; Negative regulation of Wnt signaling pathway</td>
</tr>
</tbody>
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<td>15 vs. 5</td>
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<tr>
<td></td>
<td></td>
<td>FXR KO vs. WT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2F8</td>
<td>E2F transcription factor 8</td>
<td>Up</td>
<td>Unfavored</td>
<td>Regulates progression from G1 to S phase by ensuring the nucleus divides at the proper time (34)</td>
</tr>
<tr>
<td>TREM2</td>
<td>triggering receptor expressed on myeloid cells 2</td>
<td>Up</td>
<td>Unfavored</td>
<td>Functions in immune response and may be involved in chronic inflammation by triggering the production of constitutive inflammatory cytokines</td>
</tr>
<tr>
<td>MTHFD1L</td>
<td>methylenetetrahydrofolate dehydrogenase (NADP + dependent) 1 like</td>
<td>Up</td>
<td>Unfavored</td>
<td>Involved in the synthesis of tetrahydrofolate (THF) in the mitochondrion. THF is important in the de novo synthesis of purines and thymidylate and in the regeneration of methionine from homocysteine.</td>
</tr>
<tr>
<td>PCK1</td>
<td>phosphoenolpyruvate carboxykinase 1</td>
<td>Down</td>
<td>Favored</td>
<td>A main control point for the regulation of gluconeogenesis (36)</td>
</tr>
<tr>
<td>TSC22D3</td>
<td>TSC22 domain family member 3</td>
<td>Down</td>
<td>Favored</td>
<td>Function as transcriptional regulators in the anti-inflammatory and immunosuppressive effects of this steroid and chemokine</td>
</tr>
<tr>
<td>SLC22A7</td>
<td>solute carrier family 22 member 7</td>
<td>Down</td>
<td>Favored</td>
<td>Involved in the sodium-independent transport and excretion of organic anions, some of which are potentially toxic.</td>
</tr>
<tr>
<td>CYP2U1</td>
<td>cytochrome p450 family 2 subfamily U member 1</td>
<td>Down</td>
<td>Favored</td>
<td>Xenobiotic metabolic process; Cell death; Arachidonic acid metabolic process</td>
</tr>
</tbody>
</table>

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### Table 1: Gene expression and metabolite changes in HCC

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>SARDH</td>
<td>sarcosine dehydrogenase</td>
<td>Down</td>
<td>Favored</td>
<td>An enzyme localized to the mitochondrial matrix which catalyzes the oxidative demethylation of sarcosine</td>
</tr>
<tr>
<td>TTC36</td>
<td>tetratricopeptide repeat domain 36</td>
<td>Down</td>
<td>Favored</td>
<td>May function as a tumor suppressor in hepatocellular carcinoma (HCC) since it promotes apoptosis but is downregulated in HCC.</td>
</tr>
<tr>
<td>APOC1</td>
<td>apolipoprotein C1</td>
<td>Down</td>
<td>Favored</td>
<td>High-density lipoprotein and very low-density lipoprotein metabolism; Inhibits cholesteryl ester transfer protein in plasma</td>
</tr>
</tbody>
</table>

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Additionally, 44 hepatic metabolites (e.g., leucine, urea, ribitol, palmitic acid, oxalic acid, myo-inositol, succinic acid, ethanolamine, uracil, sorbitol, galactitol, and taurine) were commonly altered by WD, aging, and FXR deactivation (Fig. S16). To further identify liver disease-related metabolites or gut microbes, an association analysis was performed between hepatic features (76 transcripts and 44 metabolites) and non-hepatic features (serum/urine metabolites and cecal microbiota at genus level) (Fig. S17). An expanded heatmap revealed the expressions of those 25 hepatic transcripts, which predict human HCC survival (Table 1), correlated with serum or urine metabolites and microbes (Fig. S17). The 18 genes whose upregulation predicts worse survival of HCC patients (red) were positively correlated with serum N-methylhydantoin and ornithine, as well as cecal *Dehalobacterium, Bacteroides*, and *Desulfovibrio*. The 7 genes whose downregulation predicts worse survival of HCC patients (blue) were positively associated with serum concentrations of formate, arginine, betaine, and glycolate.

### Discussion

This study investigated the impact of WD intake and aging on metabolic liver disease development in WT and FXR KO mice based on multi-omics data. It revealed the indispensable roles of FXR in regulating age and diet-associated liver metabolism. Common and unique hepatic transcripts, liver, serum, and urine metabolites, as well as cecal microbiota affected by diet, and/or age, and/or FXR deactivation were
uncovered to identify metabolic features for the healthy liver, NAFLD, and HCC. Those molecular signatures help our understanding of the mechanisms by which metabolic liver diseases develop. They can also be early diagnostic markers.

WD-fed WT mice have metabolic perturbations with mild NASH [21]. Transcriptomic data revealed that the cholesterol biosynthesis pathway was one of the key pathways inhibited by WD intake in both WT and FXR KO mice. WD upregulated genes involved in the regulation of cytokine production but downregulated genes in OXPHOS in WT mice. Consistent with our previous findings, prolonged WD intake led to hepatic lymphocyte and neutrophil infiltration when mice reached 10 months of age [5]. Furthermore, many proinflammatory cytokine and chemokine genes including the Ccl17, Ccl20, Ccl2, Tnf, and Il6 were induced in 15-months old mice revealing combined impact of diet and age [4]. Reduced OXPHOS, which indicates mitochondrial dysfunction, together with heightened inflammation contribute to deregulated lipid and glucose metabolism, insulin resistance, and cancer development [22–24].

In FXR KO mice, WD intake increased serum alanine aminotransferase at 5 months of age indicating liver injury [14]. WD uniquely enhanced the expression of iron binding related genes in FXR KO mice. Iron overload leads to liver injury through the production of reactive oxygen species, which is implicated in liver carcinogenesis [25]. However, the exact role of FXR in regulating iron remains to be studied.

PCA of hepatic transcriptomics data revealed that WD intake facilitated aging, which is characterized by inflammaging. In consistency, 15-months old livers had induced cytokine, inflammatory responses, leukocyte migration, myeloid leukocyte activation, etc. Moreover, similar to WD intake, many age-related changes in hepatic transcriptomes found in WT mice were no longer noted in FXR KO mice. OXPHOS was the top downregulated pathway caused by WD intake as well as aging in WT, but not in FXR KO mice. These findings clearly indicate the pivotal role of FXR in regulating OXPHOS, the process to generate ATP in mitochondria. Along the same line, 15-months old livers had reduced mitochondrial transmembrane transport, energy coupled proton transport, and mitochondrion organization. Together, our data suggest that activation of FXR may be a way to alleviate aging and diet reduced OXPHOS.

By examining the differential expression of hepatic genes between WT and FXR KO mice in healthy diet-fed mice, the specific roles of FXR became apparent. FXR dictates neuron differentiation, muscle contraction, organ growth, and actin cytoskeleton organization in addition to metabolism. Moreover, WD intake further enriched the chemical carcinogenesis, extracellular matrix, and blood vessel development pathways in FXR KO mice leading to tumorigenesis.

There were 654 hepatic transcripts commonly altered with differential expressions due to diet intake, ages, and FXR deficiency. Those transcripts have the potential to be metabolic disease development signatures. Among them, 76 transcripts have been documented to be differentially expressed in human HCC vs. healthy livers. Moreover, 25 out of those 76 transcripts can predict the overall survival rate of HCC patients (Table 1). Moreover, human transcripts for Trim59, Tpx2, Kif20a,Hmmr, and Nuf2 are known biomarkers of HCC [26–30]. Additionally, it has been shown that Kif18b, Top2a, Ect2, E2f8, and Aspm upregulations promote human HCC progression [31–35]. In contrast, Pck1, Tsc22d3, Ttc36,
Slc22a7, Cyp2u1, Sardh, and Apoc1, whose expressions are downregulated due to WD intake, aging, and FXR KO, are reduced in human HCC. Furthermore, overexpression of the Pck1 gene protects against HCC via activating gluconeogenesis and inhibiting glycolysis pathways [36]. These observations revealed the relevance of our data to human HCC. Together, the generated data uncovered additional hundreds of biomarkers which may be early markers for HCC.

Metabolites can impact hepatic inflammation and metabolic disease development. In the liver, irrespective of ages, WD increased uracil, oxoproline, and myo-inositol but decreased linoleic acid. Uracil and oxoproline (a cyclized derivative of L-glutamic acid) are etiologic factors for NASH [37]. Myo-inositol is a sugar alcohol and a precursor of inositol triphosphate, acting as an intracellular second messenger and regulating hormonal signaling including insulin [38, 39]. In consistency, linoleic acid is decreased in aging mice and human HCC [40]. In addition, WD enriched steroid biosynthesis with increased squalene in WT mice, and the accumulation of squalene in the liver decreases hepatic cholesterol and triglycerides [41]. Citrate cycle (TCA cycle) and glycolysis/gluconeogenesis, enriched by WD only in FXR KO mice, are associated with type 2 diabetes [42].

Hepatic alanine, aspartate, and glutamate metabolism is one of the most significant pathways altered by aging in WT but not in FXR KO mice. It is also the top pathway deregulated by FXR deactivation. Those amino acid metabolic pathways might be involved in the pathogenesis of metabolic syndrome [43]. In addition, aging decreased ethanolamine (aminoalcohol) and metabolites involved in the aminoacyl-tRNA biosynthesis in WT mice. Ethanolamine is a precursor for phospholipids synthesis. Dietary ethanolamine exhibits a protective effect against hyperlipidemia in aged mice [44]. Reduced hepatic ethanolamine was consistently found in WD-fed WT mice further suggesting its protective role. Moreover, ursodeoxycholic acid, which was reduced by WD and FXR KO, has hepatoprotective effects partly by regulating aminoacyl-tRNA biosynthesis [5, 45]. Thus, our data suggest the significance of hepatic amino acid metabolism in aging and FXR dependency.

By comparing FXR KO with WT mice, FXR is essential for propanoate metabolism (reduced hepatic 2-hydroxybutyric acid, increased 2-ketobutyric acid, succinic acid, and beta-alanine). 2-Hydroxybutyric acid is produced by threonine and methionine as well as glutathione anabolism. It metabolizes into propionyl-CoA, a coenzyme A derivative of propionic acid, which converts into succinyl CoA and participates in the TCA cycle and gluconeogenesis. 2-Hydroxybutyric acid is functionally related to butyric acid which is associated with gut microbiota, and vancomycin pretreatment increases serum 2-hydroxybutyric acid [46]. It has been shown that 2-hydroxybutyric acid via intraperitoneal injection protects against acetaminophen-induced liver injury in mice [46]. In contrast, succinic acid has been identified as an oncometabolite in causing cancer [47]. Consistently, the highest concentration of hepatic succinic acid was observed in WD-fed FXR KO at 15 months of age. Therefore, deregulated hepatic propanoate metabolism contributes to FXR KO-induced hepatocarcinogenesis.

FXR KO mice have reduced hepatic melibiose in a diet-independent manner. Melibiose is a nondigestable disaccharide formed by an α-1,6 linkage between galactose and glucose. Cleaved by α-galactosidase
found in *Saccharomyces pastorianus*, melibiose breaks down into glucose and galactose. Elevated hepatic melibiose found in FXR KO mice reveals the significance of FXR in the regulation of sugar metabolism via the gut-liver axis. Specific to WD-fed animals, FXR KO induces hepatic orotic acid and 3-phosphoglycerate, which have been implicated in tumorigenesis [48, 49].

It is interesting to note that altered hepatic arginine biosynthesis is shared by dietary intervention, aging, and FXR KO. Arginine is synthesized from citrulline in the urea cycle, which is an energetically costly process, and further metabolized into urea. Increased urea was consistently found in the livers of aged mice. Urea is derived from ammonia, which is generally considered to play a role in hepatic encephalopathy [50]. The potential roles of the ammonia-urea cycle in metabolic disease development and liver injury warrant further investigation. By contrast, hepatic fumaric acid (dicarboxylic acid) involved in arginine biosynthesis was increased by both WD and FXR KO. Fumaric acid is formed by the oxidation of succinic acid and a precursor of L-malate in the TCA cycle. In consistency, malic acid was also elevated in WD-fed FXR KO mice. Fumaric acid has been identified as a cancer-causing metabolite [51], indicating TCA cycle involves in the pathogenetic arginine biosynthesis during metabolic liver disease development (Fig. S18).

Serum metabolomics can distinguish chronological ages in both genotypes. Increased amino acids (alanine, isoleucine) as well as decreased 1.3-dihydroxyacetone (DHA, non-toxic sugar) and ketone bodies (acetone, acetoacetate) in serum were noted in aged mice. Moreover, aging modulated the TCA cycle (reduced glucose, increased pyruvate and succinate) in both genotypes. In consistency with the serum data, liver alanine and isoleucine were increased with age. Alanine is produced from pyruvate by transamination. Isoleucine is a branch-chain amino acid (BCAA), and circulating BCAA increases in humans with metabolic diseases including obesity and type 2 diabetes [52].

In contrast with serum metabolome, urine metabolome distinguished dietary effects in both genotypes based on the PCA data. Reduced urine TMA and trimethylamine N-oxide (TMAO) are the signatures of WD intake. TMAO is synthesized endogenously from TMA, which is generated from microbial metabolism of choline, lecithin, or carnitine. TMA via the portal circulation converts into TMAO in the liver. A high-fat diet can reduce urine TMA/TMAO, and TMAO supplementation aggravates liver steatosis by inhibiting FXR [15, 53]. Consistent with WD effects, FXR KO mice also had reduced urine TMA.

Knockout of FXR leads to metabolic deteriorations in urine metabolomics as well. Irrespective of diets and ages, FXR KO increased urine 2-hydroxyvalerate, which is increased with lactic acidosis occurring in succinic acidemia [54]. It is in line with elevated hepatic succinic acid in FXR KO mice which are cancer prone. In contrast, FXR KO reduced urine taurine and creatinine. Taurine is an essential amino acid that conjugates bile acids. The reduced output might suggest increased demand in conjugation. Creatinine is a waste product and its reduction in urine might be due to inefficient kidney/glomerular filtration.

PCA showed that the cecal microbiota community structure was distinctly different based on ages in WT but not in FXR KO mice, indicating FXR has an indispensable role in regulating age-associated gut microbiota. *Erysipelotrichaceae* and *Lachnospiraceae* (Fig. S9), as butyrate-producing bacteria, were
reduced in aged WT mice. Age associated reduction of *Rikenellaceae* was unique for FXR KO mice, the reduced *Rikenellaceae* is also found in metabolic disorders including NAFLD [55]. FXR KO mice also have a distinct gut microbiota structure in comparison to the WT, and *Bacteroidaceae* are expanded in FXR KO mice [4]. We further showed that *Bacteroides* at genus level under the *Bacteroidaceae* family was consistently increased due to lack of FXR irrespective of diets and ages. Taken together, FXR is necessary for maintaining eubiosis and hepatic metabolism.

Using the data generated from the non-liver specimens, association analysis (Fig. S17) revealed that reduced serum betaine was linked with the elevated expression of genes implicated in HCC poor survival. Serum betaine, reduced in FXR KO mice, is an antioxidant that inhibits inflammation and apoptosis but upregulates cytoprotective Akt/mTOR signaling in fatty liver disease [56]. Moreover, expanded *Dehalobacterium, Bacteroides,* or *Desulfovibrio* were also linked with high expression levels of genes implicated in HCC poor survival. It has been shown that *Dehalobacterium, Bacteroides,* and *Desulfovibrio,* which increased in FXR KO mice, contribute to metabolic liver disease development in the mouse model [57, 58].

**Conclusions**

Collectively, FXR can be a target for improving human HCC survival, and metabolites or bacteria from noninvasive specimens can be used for the early detection markers of metabolic liver diseases.

**Abbreviations**

BA, bile acid; CD, control diet; DEGs, differentially expressed genes; FXR, farnesoid X receptor; GPBAR1, G protein-coupled bile acid receptor 1; HCC, hepatocellular carcinoma; KO, knockout; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; OXPHOS, oxidative phosphorylation; PCA, principal component analysis; TCGA, The Cancer Genome Atlas; TMA, trimethylamine; TMAO, trimethylamine N-oxide; WD, Western diet; WT, Wild-type

**Declarations**

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Availability of data and materials

RNA sequencing data are available on Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) (GSE216375). All data to support the conclusions are present in the paper and supplemental Figures/Tables. Additional information related to this paper can be requested from the authors.

Conflict of interests

The authors declare no competing financial interests.

Ethics approval and consent to participate

This study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

Consent for publication

No individual person's data were used in this study.

References


Figures
Figure 1

The effects of diets on hepatic transcriptomes of WT and FXR KO mice with different ages. (A) Principal component analyses of liver transcriptomes of 5-, 10-, and 15-months old WT and FXR KO mice fed with either a CD or WD since weaning. (B) Venn diagrams show the numbers of genes differentially expressed due to diet (WD vs. CD) intake in WT or FXR KO mice. (C) Pathway enrichment analyses for up and downregulated genes affected by differential diet intake using Metascape. The top 20 pathways are
shown (adjusted \( p \) value < 0.05). WD-regulated pathways shared by WT and FXR KO mice are in purple. The numbers of differentially expressed genes in each pathway are shown in the bars.

Figure 2

The effects of diets on hepatic metabolomes in WT and FXR KO mice. (A) Principal component analyses of liver metabolomes in WT and FXR KO mice (B) Venn diagrams show the numbers of distinct and
overlapping metabolites that were changed by differential diet intake in 3 age groups (raw $p$ value <0.05 and FDR <0.1). (C) Quantitative enrichment analyses of diet altered metabolites. The top 10 pathways are shown ($p$ value <0.05). Common metabolic changes in metabolites and pathways found in both genotypes are marked in purple.

Figure 3
The effects of ages on hepatic transcriptomes in WT and FXR KO mice on different diets. (A) Principal component analyses of liver transcriptomes in WT and FXR KO mice fed with either a CD or WD. (B) Venn diagrams show the numbers of genes differentially expressed due to age (15 vs. 5) in WT or FXR KO mice (fold change ≥ 2 and adjusted p value < 0.05). (C) Pathway enrichment analyses for up and downregulated genes affected by age based on Metascape. The top 20 pathways are shown (adjusted p value < 0.05). Age-regulated pathways shared by WT and FXR KO mice are in purple. The numbers of differentially expressed genes in each pathway are shown in the bars.
Figure 4

The effects of ages on hepatic metabolomes in WT and FXR KO mice. (A) Principal component analyses of liver metabolomes of WT and FXR KO mice. (B) Venn diagrams show the numbers of distinct and overlapping metabolites that were changed by age (raw $p$ value <0.05 and FDR <0.1). (C) Quantitative enrichment analyses of age altered metabolites. The top 10 pathways are shown ($p$ value <0.05). Common metabolic changes in metabolites and pathways found in both WT and FXR KO mice are marked in purple.
Figure 5

The effects of FXR KO on hepatic transcriptomes. (A) Principal component analyses of liver transcriptomes of WT and FXR KO mice fed with differential diets until 5, 10, and 15 months old. (B) Venn diagrams show the numbers of genes differentially expressed due to FXR KO (fold change ≥ 2 and adjusted p value < 0.05). (C) Pathways and numbers of genes that were up or down regulated by FXR KO found in both CD- and WD-fed mice based on Metascape. The top 20 pathways are shown (adjusted p
value <0.05). Common and specific transcriptomic changes (pathways and numbers of differentially expressed genes in each pathway) due to FXR KO in CD- and WD-fed mice.

Figure 6

The effects of FXR KO on hepatic metabolomes. (A) Principal component analyses of liver metabolomes of WT and FXR KO mice fed with differential diets until 5, 10, and 15 months old. (B) Venn diagrams
show the numbers of distinct and overlapping metabolites that were altered by FXR KO in CD- and WD-fed mice (raw p value <0.05 and FDR <0.1). (C) Quantitative enrichment analyses of FXR KO altered metabolites. The top 10 pathways are shown (p value <0.05). Common enriched pathways in both CD- and WD-fed mice are marked in purple.

Figure 7
Common changes of hepatic transcripts due to differential diet intake, ages, and FXR KO in association with the expression of genes implicated in human HCC patient survival. (A) Venn diagram shows the numbers of common genes (transcriptomes) (fold change ≥ 2 and adjusted $p$ value < 0.05). (B) A heatmap reveals transcript levels of mouse genes implicated in hepatic metabolic disease (n = 76) and human genes differentially expressed in human HCC and healthy livers. (C) Pathway enrichment analyses of 76 mouse hepatic genes that are implicated in human HCC survival using Metascape (adjusted $p$ value < 0.05). The numbers of differentially expressed genes in each pathway are shown in the bar graph. LIHC, liver hepatocellular carcinoma

Supplementary Files

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