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Impact of Microbiome on Hepatic Metabolizing Enzymes and Transporters in Mice during Pregnancy[S]

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ABSTRACT

The microbiome and pregnancy are known to alter drug disposition, yet the interplay of the two physiologic factors on the expression and/or activity of drug metabolizing enzymes and transporters (DMETs) is unknown. This study investigated the effects of microbiome on host hepatic DMETs in mice during pregnancy by comparing four groups of conventional (CV) and germ-free (GF) female mice and pregnancy status, namely, CV nonpregnant, GF non-pregnant, CV pregnant, and GF pregnant mice. Transcriptomic and targeted proteomics of hepatic DMETs were profiled by using multiomics. Plasma bile acid and steroid hormone levels were quantified by liquid chromatography tandem mass spectrometry. CYP3A activities were measured by mouse liver microsome incubations. The trend of pregnancy-induced changes in the expression or activity of hepatic DMETs in CV and GF mice was similar; however, the magnitude of change was noticeably different. For certain DMETs, pregnancy status had paradoxical effects on mRNA and protein expression in both CV and GF mice. For instance, the mRNA levels of *Cyp3a11*, the murine homolog of human *CYP3A4*, were decreased by 1.7-fold and 3.3-fold by pregnancy in CV and GF mice, respectively; however, the protein levels of CYP3A11 were increased similarly ∼twofold by pregnancy in both CV and GF mice. Microsome incubations revealed a marked induction of CYP3A activity by pregnancy that was 10-fold greater in CV mice than that in GF mice. This is the first study to show that the microbiome can alter the expression and/or activity of hepatic DMETs in pregnancy.

SIGNIFICANCE STATEMENT

We demonstrated for the first time that microbiome and pregnancy can interplay to alter the expression and/or activity of hepatic drug metabolizing enzymes and transporters. Though the trend of pregnancy-induced changes in the expression or activity of hepatic drug metabolizing enzymes and transporters in conventional and germ-free mice was similar, the magnitude of change was noticeably different.

Introduction

Drug safety in pregnant women is a critical issue for drug development and regulatory agencies because of observed changes in efficacy and toxicity of medications during pregnancy (Beigi et al., 2016; Food and Drug Administration, 2018). Understanding how drug disposition is changed in pregnant women is therefore of utmost importance (see below). However, there remain gaps in knowledge of other factors that can affect drug disposition in this vulnerable population. The impact of the microbiome on drug disposition could be one such example (Sharma et al., 2019).

Pregnancy changes the maternal body in numerous ways to accommodate the developing fetus. Increased total body volume, decreased plasma albumin, increased cardiac output and renal glomerular filtration rate, and altered expression or activity of drug metabolizing enzymes and transporters (DMETs) profoundly alter drug disposition in pregnant women versus nonpregnant women or men (Helldén and Madadi, 2013; Isoherranen and Thummel, 2013; Pariente et al., 2016; Tasnif et al., 2016). Previous studies have shown increased activity of CYP3A4, CYP2D6, and CYP2C9 and decreased activity of CYP2C19 and CYP1A2 by using respective probe substrates prescribed to women during pregnancy (Feghali et al., 2015). Drug transporters also could have dynamic activity or mRNA expression changes during

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ABBREVIATIONS: Abc, ATP-binding cassette transporter family; BSA, bovine serum albumin; CA, cholic acid; CDCA, chenodeoxycholic acid; CV, conventional; CVNP, conventional nonpregnant; CVP, conventional pregnant; DCA, deoxycholic acid; DMET, drug metabolizing enzyme and transporter; FDR, false discovery rate; FXR, farnesoid X receptor; gd, gestation day; GF, germ-free; GFNP, germ-free nonpregnant; GFP, germ-free pregnant; Gst, glutathione *S*-transferases; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; LC-MS/MS, liquid chromatography tandem mass spectrometry; α/β-MCA, α/β-murine cholic acid; ω-MCA, ω-muricholic acid; MDCA, murideoxycholic acid; miRNA, microRNA; Mrp3/Abcc3, multidrug resistance–associated protein 3; Oat2, organic anion transporter; P450, cytochrome P450; PXR, pregnane X receptor; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; RNA-seq, RNA sequencing; Slc, solute carrier family; Slco, solute carrier organic anion transporter family; Sult, sulfotransferases; UDCA, ursodeoxycholic acid; Ugt, UDP-glucuronosyltransferases.

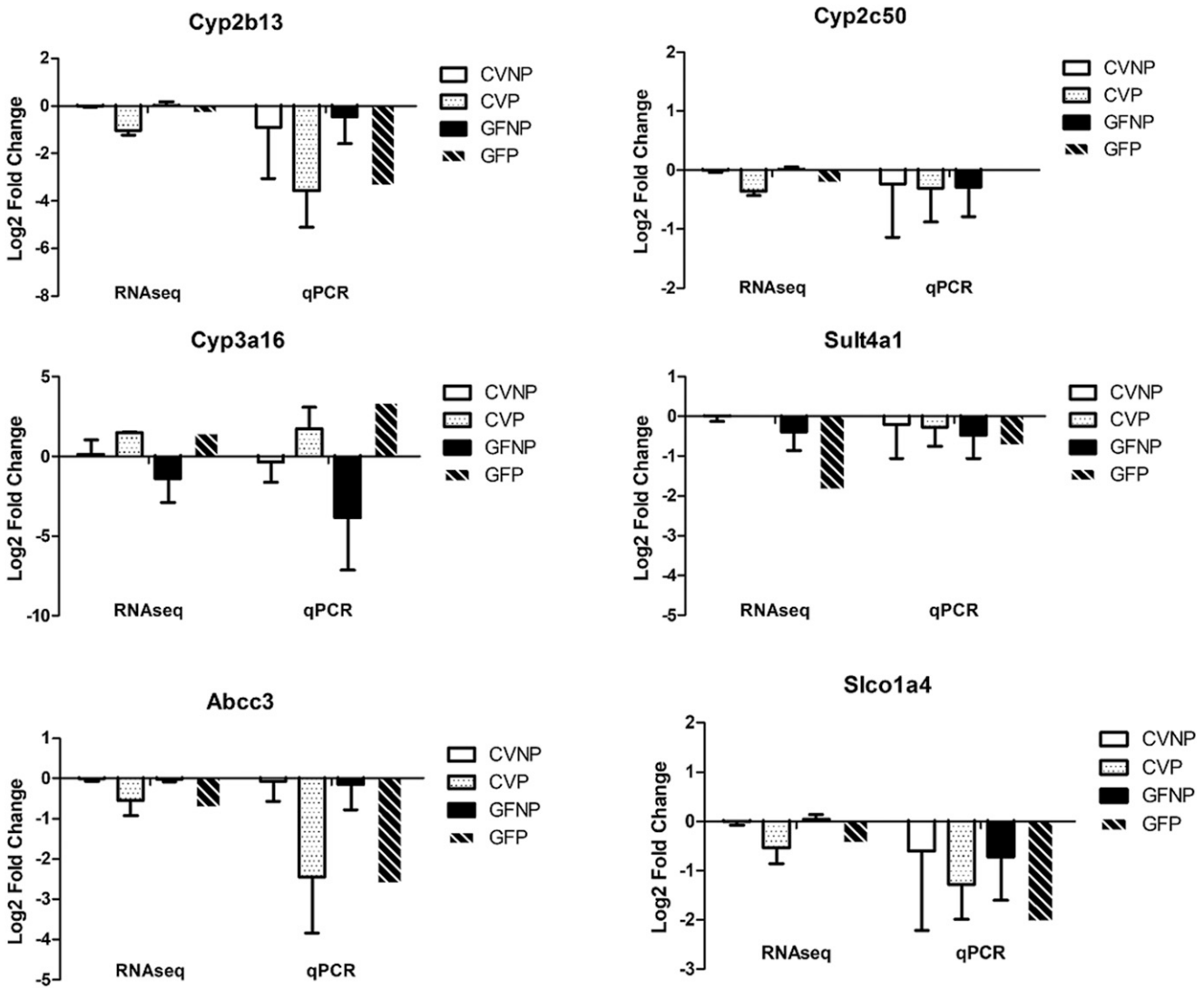

Fig. 1. Comparison of expression of selected genes determined by RNA-seq and qRT-PCR analysis. Log twofold change is relative to the CVNP group for both RNA-seq and qRT-PCR data. Shown are means ± S.D. of gene expression data from five to six different mouse liver tissues.

pregnancy. For example, the activity of P-glycoprotein and organic cation transporter 2 in the kidney appears to be induced during human pregnancy, whereas mRNA expression of multidrug resistance protein 3 (Mrp3/Abcc3) in the mouse liver is downregulated by pregnancy (Isoherranen and Thummel, 2013; Shuster et al., 2013). Pregnancy-related hormones such as progesterone, estradiol, and cortisol, which are ligands of nuclear receptors such as glucocorticoid receptor and pregnane X receptor (PXR), have been proposed to mediate induction of DMETs during pregnancy (Kliewer et al., 1998; Dussault et al., 2003; Papacleovoulou et al., 2011). Therefore, a mechanistic understanding of the changes in expression or activity of DMETs during pregnancy will help optimize the dosing regimens of drugs for improved efficacy and safety for both the mother and her fetus.

The microbiome is often referred to as the second human genome because of the abundance and diversity of bacteria. The microbiome can change drug disposition and toxicity profiles by directly metabolizing and inactivating compounds (Spanogiannopoulos et al., 2016). Indirectly, gut bacteria can alter drug disposition by changing the expression or activity of host DMETs (Spanogiannopoulos et al., 2016). It has been proposed that the predominant mechanism by which bacteria modulate DMET expression is by the modification of secondary bile acids (Claus et al., 2011; Klaassen and Cui, 2015). Primary bile acids are synthesized in the liver from cholesterol and subsequently transported into the bile duct by efflux transporters, such as the bile salt export pump. The bile duct leads primary bile acids into the duodenum where intestinal bacteria deconjugate, dehydroxylate, and epimerize them into secondary bile acids. Intestinal bacteria such as *Clostridia* from Firmicutes phylum express the enzymes (e.g., hydroxysteroid dehydrogenase and 7-dehydratase) necessary for these biotransformation reactions (Ridlon et al., 2006). The primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) as well as secondary bile acid deoxycholic acid (DCA), and their taurine conjugated forms have been shown to activate farnesoid X receptor (FXR), which regulates bile acid production (Wang et al., 1999; Kong et al., 2012). Lithocholic acid (LCA) has been shown to activate PXR (Staudinger et al., 2001), which in turn upregulates the expression of certain cytochrome P450s (P450). In mice, it has been demonstrated that the primary muricholic acids are antagonists of FXR, and thus the lack of transformation of primary bile acids into secondary bile acids in GF mice would result in accumulation of FXR antagonists (primary bile acids) and reduction of PXR agonists (secondary bile acids); ultimately, this decreases FXR and PXR signaling in the intestine and the liver (Klaassen and Cui, 2015;

TABLE 1

Pregnancy and the microbiome alter mRNA expression of key DMETs in female C57BL/6 livers

This list of genes was generated using the following filtration criteria: fold-change >1.5 or <0.65 and FDR <0.1, on at least one comparison group between CVP and CVNP, GFP and GFNP, GFNP and CVNP, or GFP and CVP. Statistically significant differences with FDR values of <0.1 are highlighted in bold.

Gene Symbol	CVP vs. CVNP		GFP vs. GFNP		GFNP vs. CVNP		GFP vs. CVP	
	Fold Change	FDR	Fold Change	FDR	Fold Change	FDR	Fold Change	FDR
Cyp17a1	1.7	0.28	**2.9**	**0.01**	0.9	1	1.6	0.704
Cyp26a1	**3.2**	**0.076**	**4.2**	**0.02**	0.9	1	1.1	0.996
Cyp26b1	**<0.1**	**<0.001**	**0.1**	**<0.001**	1.9	0.982	5	0.289
Cyp2b13	**0.1**	**<0.001**	**0.3**	**<0.001**	1.2	1	**5.3**	**<0.001**
Cyp2c37	**0.3**	**0.002**	0.6	0.152	1	1	1.8	0.401
Cyp2c38	**0.4**	**0.007**	1	0.965	1.3	1	**3**	**0.013**
Cyp2c39	1	0.983	**2.4**	**0.022**	1.1	1	2.7	0.154
Cyp2c50	**0.2**	**<0.001**	**0.5**	**0.001**	1	1	**2**	**0.066**
Cyp2c54	**0.4**	**0.001**	0.8	0.561	1	1	**2.2**	**0.074**
Cyp2c55	0.5	0.216	**0.1**	**<0.001**	2.8	0.319	0.8	0.974
Cyp2c67	**0.4**	**0.012**	0.6	0.279	1.4	1	2.3	0.238
Cyp2c69	1	0.998	**2.6**	**0.02**	0.6	0.982	1.6	0.708
Cyp2d40	**3.9**	**<0.001**	**5.4**	**<0.001**	0.7	1	1	1
Cyp2d9	**0.3**	**0.025**	**0.2**	**0.002**	1.8	1	1.1	1
Cyp2g1	**2.8**	**0.047**	**3.6**	**0.01**	0.6	1	0.8	0.964
Cyp39a1	**0.4**	**0.031**	**0.3**	**0.017**	1	1	0.9	0.999
Cyp3a11	0.6	0.29	**0.3**	**0.005**	0.6	0.953	0.3	0.131
Cyp3a16	**20.6**	**0.001**	**128**	**<0.001**	0.2	0.578	1.4	0.953
Cyp3a41a	**4.8**	**0.047**	**10.8**	**0.002**	0.6	1	1.4	0.941
Cyp3a41b	**74.2**	**<0.001**	**181.7**	**<0.001**	0.5	1	1.3	0.989
Cyp3a44	**14.3**	**<0.001**	**30.5**	**<0.001**	0.8	1	1.7	0.816
Cyp3a63-ps	**5.1**	**0.047**	**8.5**	**0.007**	0.9	1	1.5	0.926
Cyp4a14	**0.4**	**0.021**	**0.3**	**0.002**	1.2	1	0.9	0.958
Cyp4a31	**4.6**	**<0.001**	**5.3**	**<0.001**	1.1	1	1.3	0.909
Cyp4f13	**0.6**	**0.042**	0.8	0.329	0.8	1	1.1	0.987
Cyp4f15	**0.5**	**0.033**	**0.5**	**0.052**	1	1	1.1	0.976
Ugt1a10	0.9	0.977	**9.6**	**0.004**	0.2	0.439	2.4	0.689
Ugt1a5	**2.6**	**0.062**	**3.3**	**0.016**	0.5	0.949	0.7	0.833
Ugt1a6b	**0.3**	**0.042**	**0.3**	**0.023**	1	1	0.9	0.999
Ugt2b38	0.2	0.139	**<0.1**	**0.013**	0.5	1	0.1	0.408
Sult2a7	**0.2**	**0.061**	**0.2**	**0.031**	0.9	1	0.7	0.964
Sult3a1	**3.6**	**0.056**	**12.9**	**<0.001**	0.3	0.359	1	1
Sult3a2	**53.9**	**<0.001**	**226.7**	**<0.001**	0.2	0.605	0.9	1
Sult4a1	**287.7**	**<0.001**	3.5	0.333	5.2	1	0.1	0.129
Gsta2	0.6	0.215	**0.4**	**0.033**	2	0.605	1.5	0.782
Gstk1	**0.5**	**0.005**	**0.5**	**0.018**	1.1	1	1.2	0.901
Gstm2	**1.8**	**0.041**	1.4	0.248	1.4	1	1.1	0.984
Gstm3	**3.1**	**0.044**	0.6	0.456	2.5	0.564	0.5	0.625
Gstp1	**3.4**	**0.002**	**2.2**	**0.039**	0.9	1	0.6	0.635
Gstp2	**5.9**	**0.016**	1.8	0.505	0.9	1	0.3	0.386
Gstt1	**0.4**	**0.005**	**0.4**	**0.017**	0.8	1	0.9	0.976
Gstt3	1.5	0.177	**2**	**0.005**	0.8	1	1.1	0.951
Abca17	**103.3**	**<0.001**	**1779**	**<0.001**	0.1	0.757	1.2	0.989
Abca5	**2**	**0.025**	**3.3**	**<0.001**	0.8	1	1.3	0.768
Abca7	0.9	0.851	**0.5**	**0.006**	1.1	1	0.6	0.298
Abcb6	**0.6**	**0.019**	0.7	0.129	1	1	1.2	0.83
Abcc3	**0.3**	**0.025**	**0.1**	**<0.001**	1	1	0.5	0.613
Abcg5	**0.4**	**0.003**	**0.4**	**0.009**	1.3	1	1.4	0.696
Abcg8	**0.5**	**0.008**	**0.6**	**0.061**	1.2	1	1.4	0.613
Slc10a1	**0.4**	**0.02**	**0.5**	**0.045**	0.8	1	1	1
Slc16a1	**2**	**0.018**	1.6	0.142	0.9	1	0.7	0.625
Slc16a6	**16.2**	**<0.001**	**14.4**	**<0.001**	1.1	1	1	1
Slc17a2	**0.3**	**0.057**	**0.2**	**0.009**	0.9	1	0.6	0.816
Slc22a15	**0.5**	**0.008**	**0.5**	**0.028**	1.1	1	1.2	0.869
Slc22a2	**260.5**	**<0.001**	**561.5**	**<0.001**	0.4	1	0.8	0.984
Slc22a7	1.3	0.713	**3.1**	**0.004**	0.5	0.437	1.1	0.975
Slc24a3	**9.5**	**<0.001**	**6.6**	**<0.001**	1.3	1	0.9	0.969
Slc25a19	0.8	0.291	**0.6**	**0.015**	1.1	1	0.9	0.857
Slc25a28	**0.6**	**0.007**	**0.7**	**0.024**	0.9	1	0.9	0.953
Slc25a30	1.7	0.128	**2.2**	**0.01**	0.5	0.315	0.7	0.639
Slc25a37	**0.5**	**0.012**	**0.6**	**0.027**	0.9	1	1	1
Slc25a51	**0.6**	**0.026**	**0.3**	**<0.001**	1.8	0.181	1.1	0.951
Slc26a10	**0.4**	**0.079**	**0.3**	**0.006**	1.3	1	0.9	0.988
Slc26a4	**0.1**	**0.009**	**0.2**	**0.016**	1.2	1	1.6	0.947
Slc27a1	0.7	0.21	**0.6**	**0.018**	1.3	1	1	1
Slc35b1	**1.8**	**0.006**	**2.5**	**<0.001**	1	1	1.3	0.617
Slc35c1	1.3	0.254	**1.6**	**0.029**	0.9	1	1.1	0.953
Slc35c2	**1.7**	**0.003**	**2**	**<0.001**	0.9	1	1	1

(*continued*)

TABLE 1—*Continued*

Gene Symbol	CVP vs. CVNP		GFP vs. GFNP		GFNP vs. CVNP		GFP vs. CVP	
	Fold Change	FDR	Fold Change	FDR	Fold Change	FDR	Fold Change	FDR
Slc36a1	**2.3**	**0.001**	**2.7**	**<0.001**	0.9	1	1	1
Slc37a1	**11.6**	**<0.001**	**6.2**	**<0.001**	1.5	1	0.8	0.946
Slc38a4	**0.3**	**<0.001**	**0.3**	**<0.001**	1.1	1	1.2	0.951
Slc39a11	**1.4**	**0.18**	**2**	**<0.001**	0.9	1	1.3	0.626
Slc39a14	**2.3**	**0.013**	**2.1**	**0.022**	1.5	0.978	1.4	0.775
Slc41a2	**30.2**	**<0.001**	**28.1**	**<0.001**	1.1	1	1	1
Slc41a3	**4.3**	**0.001**	**3.5**	**0.002**	1.9	0.781	1.5	0.753
Slc43a1	**2.6**	**0.037**	**3.1**	**0.01**	1.1	1	1.3	0.934
Slc45a3	**3**	**0.001**	**2.1**	**0.027**	1.2	1	0.9	0.948
Slc4a1	**8**	**0.002**	**24**	**<0.001**	0.9	1	2.6	0.415
Slc4a9	**0.1**	**0.025**	**0.1**	**0.001**	3.9	0.359	1.9	0.914
Slc6a9	**6.1**	**<0.001**	**9**	**<0.001**	0.7	1	1.1	1
Slc7a15	**5**	**0.102**	**19.1**	**0.01**	0.1	0.554	0.4	0.786
Slc7a2	**0.4**	**0.013**	**0.5**	**0.058**	1.3	1	1.6	0.625
Slc7a7	**5**	**<0.001**	**2.9**	**0.003**	1.1	1	0.6	0.601
Slc8b1	**0.5**	**0.023**	**0.6**	**0.055**	0.8	1	0.9	0.958
Slc9a3	0.4	0.766	**74.3**	**0.005**	0.1	1	23.5	0.238
Slco1a4	**0.4**	**0.045**	**0.4**	**0.063**	1.1	1	1.2	0.945
Slco4c1	4.8	0.519	**17.8**	**0.035**	2.6	1	9.6	0.376

Wahlström et al., 2017). Recently, it was shown that the maternal gut microbiome shifts as pregnancy progresses in humans, with an increase in the abundance of *Actinobacteria* and *Proteobacteria*, which could alter bile acid profiles and DMET expression and/or activity (Nuriel-Ohayon et al., 2016).

Although both pregnancy and the microbiome individually have a profound impact on drug disposition, no studies to date have investigated their combined effects. The objective of this study was to explore the combined effects of pregnancy and the microbiome on the expression and/or activity of hepatic DMETs using CV and GF pregnant mice. We hypothesize that the magnitude and direction of changes in expression and/or activity reflect the combined effects of pregnancy and the microbiome.

Materials and Methods

Chemicals. Liquid chromatography–mass spectrometry (LC-MS) grade methanol, water, and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ). Steroid hormone standards and deuterated internal standards for bile acids (lithocholic acid, deoxycholic acid, cholic acid, glycochenodeoxycholic acid, and glycocholic acid) were purchased from Cerilliant Corporation (Round Rock, TX) and Steraloids Inc. (Newport, RI). All other chemicals were obtained from Thermo Fisher Scientific (Rockford, IL) unless stated otherwise.

Animal Studies. Conventional C57BL/6J mice (JAX stock 000664) from The Jackson Laboratory (Bar Habor, ME) were habituated for 1 week at our own animal facility prior to experiments. Germ-free (GF) C57BL/6 colony were originated from mice purchased from the National Gnotobiotic Rodent Resource Center of the University of North Carolina at Chapel Hill. The conventional (CV) and GF mice have the same genetic background. All animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Washington (protocol 4035-04). GF mice were housed at the University of Washington Gnotobiotic Animal facility in isolators.

Both CV and GF mice were maintained on 12-hour light/dark cycles, and the same autoclaved Breeder Chow 5021 (LabDiet, St. Louis, MO), autoclaved nonacidified water, and autoclaved Enrich-N'Pure bedding (The Andersons Inc., Maumee, OH) were provided ad libitum. CV female mice at 8 weeks of age were mated with CV male mice overnight, and male mice were promptly separated from female mice in the morning. The day with the overnight housing was defined as gestation day (gd) 0. Because of difficulties in achieving pregnancies in GF mice, GF female mice of 11–15 weeks of age were mated with male mice for 72 hours prior to separation from GF male mice. Day 2 after introduction of male mice to the cage was assumed to be gd 0. For the purpose of tissue collection, all pregnant CV or GF mice were kept individually in separate cages during the period of gestation after mating. All pregnant mouse tissues were collected on gd 15, which was previously shown to be the optimal time point to observe peak levels of gene expression changes (Shuster et al., 2013). Nonpregnant female mice of similar ages (8–15 weeks) were housed exactly the same as pregnant mice as described above. On the day of tissue collection, pregnant or nonpregnant female mice were anesthetized with isoflurane, and blood was collected via cardiac puncture and immediately centrifuged (1500*g* for 10 minutes at 4°C) to isolate plasma. Tissues (e.g., liver) were removed, washed in cold saline, and immediately snap-frozen in liquid N_2. In total, plasma samples and tissues from six CV nonpregnant (CVNP), five CV pregnant (CVP), six GF nonpregnant (GFNP), and five GF pregnant (GFP) mice were collected. All samples were then stored at −80°C until analysis.

RNA-Sequencing Analysis. Total RNA was extracted from liver tissues by using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration of total RNA was determined by using a Synergy HTX Multi-mode Plate Reader (BioTek, Winooski, VT) at 260 nm. Paired-end RNA sequencing (RNA-seq) (2 × 150 bp) was performed by Novogene Bioinformatics Technology Co., Ltd. (Sacramento, CA), using the Illumina NovaSEq. 6000 with an average 20 million reads. The library was prepared by using NEBNext Ultra RNA Library Prep Kit from Illumina. We aligned reads to the mouse GRCm38.p6 transcriptome (Gencode release M19) using the *Salmon* aligner (v0.11.3) (Patro et al., 2015), and then the transcript-level counts were imported into R and summarized at the gene level by using the Bioconductor tximport package (v1.10.1) (Soneson et al., 2015). Data were subsequently filtered to remove genes that had consistently low expression levels to improve the signal-to-noise ratio by using filterByExpr function in the edgeR package (v3.24.3) implemented in R (v3.5.1). After filtering, 18,849 genes remained. Differential gene expression analysis between groups was performed by using the edgeR package with a negative binomial generalized linear model and quasilikelihood *F* tests for a given contrast (Robinson et al., 2010; Chen et al., 2016). A false discovery rate (FDR) of 10% was selected to limit the number of false positives (Benjamini and Hochberg, 1995). RNA-seq data presented in this study were deposited in the National Center for Biotechnology Information Gene Expression Omnibus data repository under accession number GSE143391.

Quantitative Real-Time Polymerase Chain Reaction. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) was performed to determine the mRNA levels of selected genes, as previously described, to validate RNA sequencing data (Shuster et al., 2013). Aliquots from the same total RNA samples used for RNA-seq analysis were transcribed to cDNAs by using random primers from High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). We selected four enzyme genes for coding (*Cyp2b13*, *Cyp2c50*, *Cyp3a16*, and *Sult4a1*) and two transporter genes (*Abcc3*

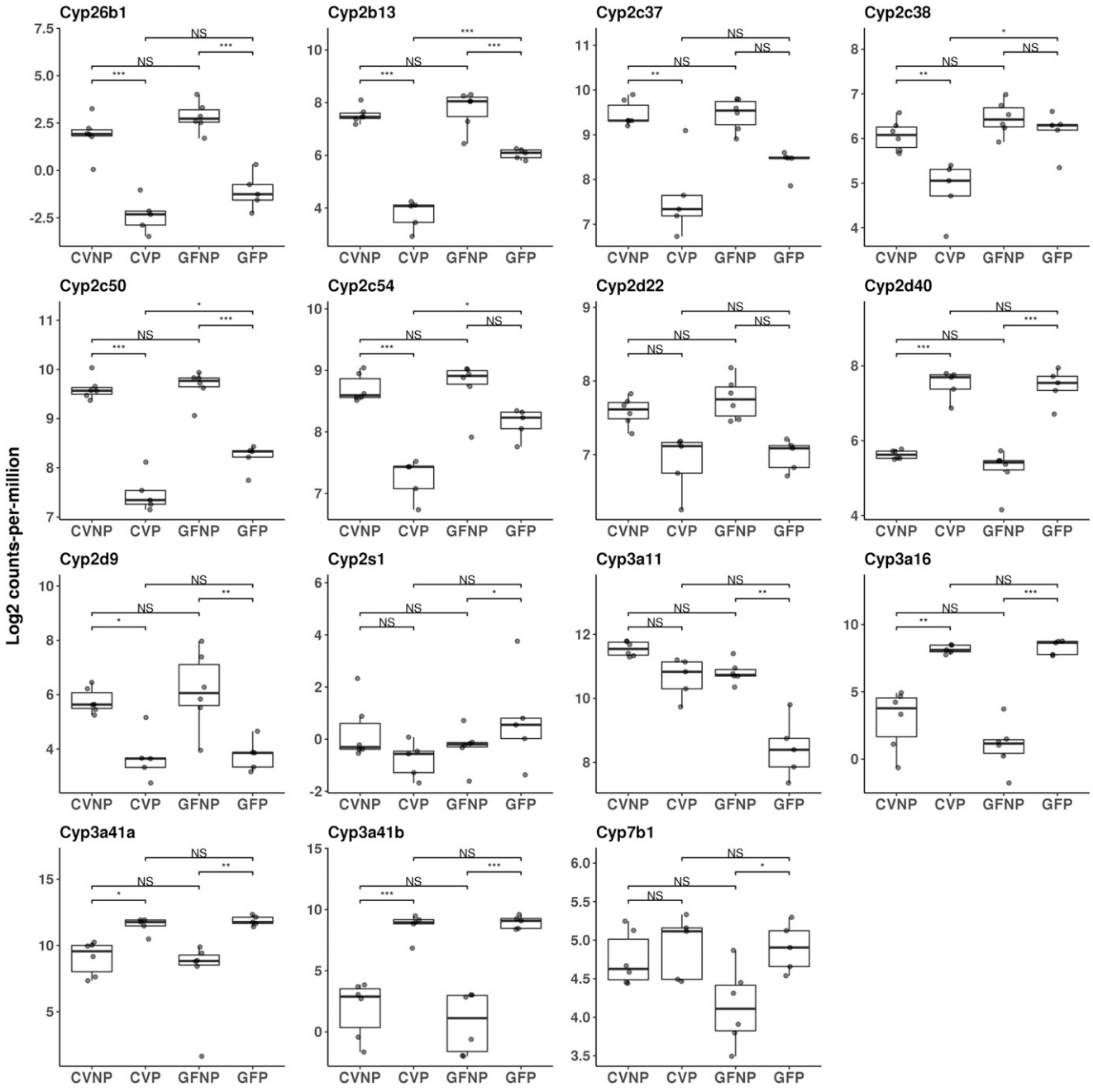

Fig. 2. Effect of pregnancy and microbiome on mRNA expression of hepatic phase I enzymes. Shown are boxplots with individual scatters of RNA-seq analysis data of hepatic phase I enzymes from female C57BL/6 mice. Data illustrates individual log2 counts per million for each DMET. All pregnant mice used were on gestation day 15. *FDR $<$ 0.1; **FDR $<$ 0.01; ***FDR $<$ 0.001.

and *Slco1a4*) for validation. The genes were selected based on the large magnitude of change in mRNA abundance observed from RNA-seq analysis. The cDNAs were amplified by using SsoAdvanced Universal SYBR Green Supermix and the Bio-Rad CFX384 Real-Time PCR Detection System (Hercules, CA). PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA). Glyceraldehyde-3-phosphate dehydrogenase was used as a housekeeping gene for normalization of the gene expression data.

Targeted Proteomic Analysis of Hepatic DMETs by LC-MS/MS. Quantification of relative protein abundance of major DMETs in the liver tissues of CV and GF mice was done by quantitative LC-MS/MS proteomic analysis using methods previously described (Bhatt and Prasad, 2018; Liao et al., 2018; Prasad et al., 2018). For P450 enzymes, changes in protein levels of CYP2C37, CYP2C50, CYP2C54, CYP2D22, CYP2D40, CYP3A11, CYP3A16, and CYP3A41 were quantified. For transporters, changes in protein levels of ABCB11, ABCB1A, ABCB1B, ABCC3, ABCG2, SLC22A2, SLC22A7, SLCO1A1, and SLCO1A4 were determined. We used the same procedures for protein isolation and digestion as previously published (Prasad et al., 2018). Briefly, approximately 50 mg of frozen liver tissue per mouse was homogenized in 2 ml of permeabilization buffer (the permeabilization and protease inhibitor solution mix) using dounce hand homogenizer. All steps were carried out on ice to minimize protein degradation. The resulting liver homogenate was shaken for 30 minutes at 4°C and then centrifuged at 16,000*g* for 15 minutes. The resulting supernatant was aliquoted for protein quantification via BCA analysis and diluted to 2 mg/ml prior to trypsin digestion. To 80 μl of post-treatment supernatant, 30 μl of ammonium bicarbonate buffer (100 mM, pH 7.8), 10 μl of human serum albumin, 20 μl of bovine serum albumin (BSA), and 10 μl of dithiothreitol were added and incubated at 95°C for 10 minutes. After cooling, 20 μl of iodoacetamide (500 mM) was added to incubate at room temperature for

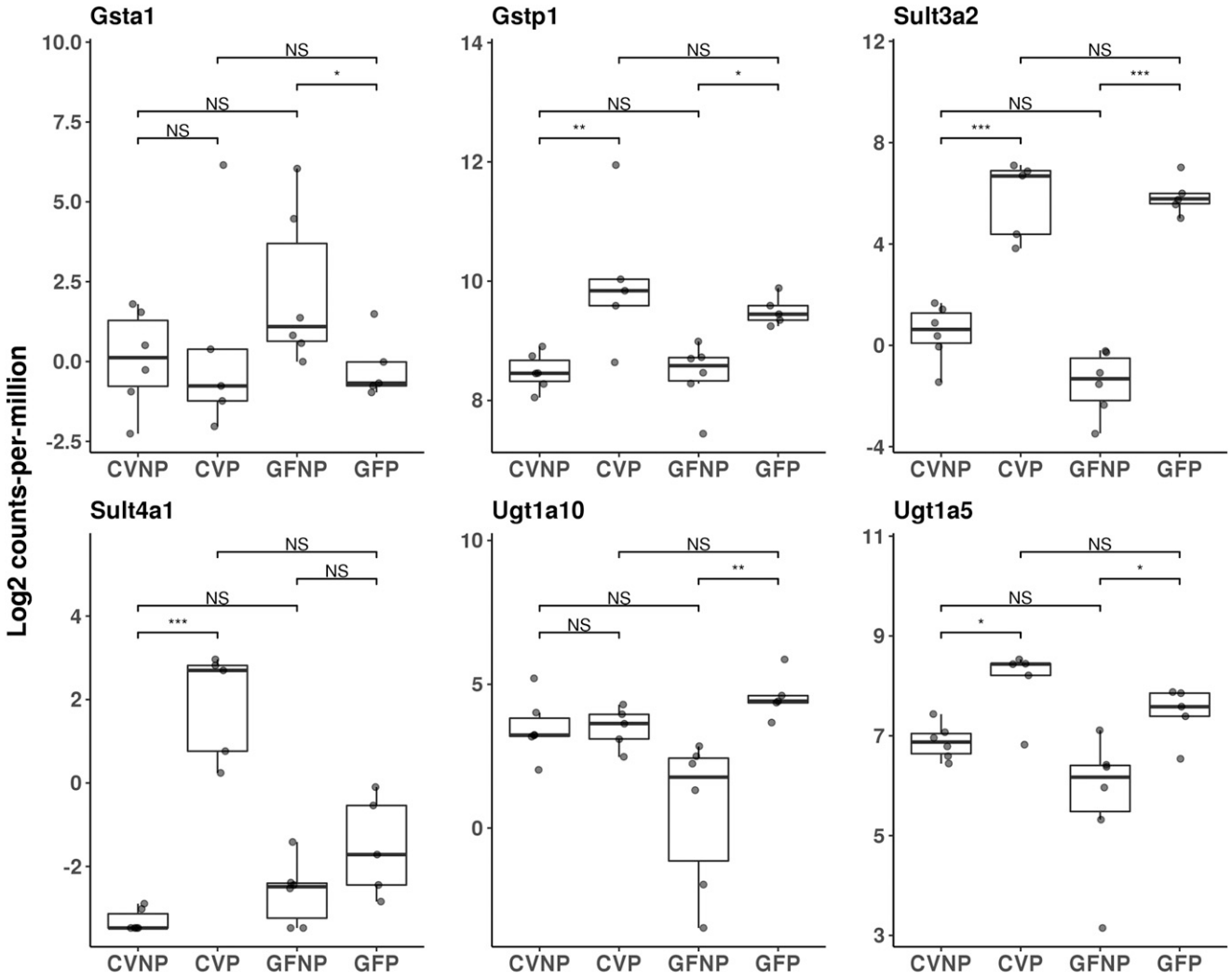

Fig. 3. Effect of pregnancy and microbiome on mRNA expression of hepatic phase II enzymes. Shown are boxplots with individual scatters of RNA-seq analysis data of hepatic phase II enzymes from female C57BL/6 mice. Data illustrates individual log2 counts per million for each DMET. All pregnant mice used were on gestation day 15. *FDR < 0.1; **FDR < 0.01; ***FDR < 0.001.

30 minutes in the dark. Then, ice-cold methanol (0.5 ml), chloroform (0.1 ml), and water (0.4 ml) were added to desalt the samples. After centrifugation at 16,000*g* for 5 minutes at 4°C, the pellet was washed with ice-cold methanol (0.5 ml) and centrifuged at 8000*g* for 5 minutes at 4°C. Trypsin [20 μl, 1:10 trypsin: protein ratio (w/w)] was added and incubated for 16 hours at 37°C while shaking at 300 rpm. The reaction was quenched with dry ice, and internal standards were added. The relative levels of protein abundance of P450 enzymes and transporter were quantified using surrogate peptides for respective P450 enzymes or transporters as standards (Supplemental Table 1). Chromatographic and mass spectrometric conditions were the same as previously described (Prasad et al., 2018). Previous studies (Bhatt and Prasad, 2018) found that it was necessary to perform various normalization steps to minimize batch-to-batch and sample-to-sample variation. Therefore, BSA and a stable-isotope–labeled heavy peptide cocktail were added as normalization controls. The quality of peptide signal was verified by plotting the correlation between two or more peptide fragments or transitions derived from different samples with the same total protein concentration. Ion suppression was assessed with multiple peptides from the same protein. This approach measures whether the variability in peak responses reflects the biologic variability. In addition, by adding BSA, an exogenous protein, into the homogenized sample before protein denaturation, the sample loss during subsequent processing steps was addressed. Moreover, the use of heavy isotope–labeled peptides as internal standard provided adjustment for possible matrix effects and sample concentration changes because of evaporation and nonspecific binding to the vials. Relative protein abundance of each enzyme or transporter was calculated by taking the average signal for all peptides that passed linearity test for each enzyme or transporter in each individual liver sample and dividing it by the average signal of the corresponding heavy isotope–labeled peptide. The ratio was subsequently normalized to BSA and the group mean (pool of all samples) of each surrogate peptide. The resulting ratio-of-ratio estimates reflect relative protein abundance of each individual enzyme or transporter. The samples used for proteomic analysis were essentially the same as those used for RNA-seq analysis or activity assay (see below) except that the number of samples used for proteomic analysis in the CVP and GFNP groups was four instead of five or six because of a lack of sufficient tissue homogenates.

Liver Microsome Isolation and Activity Assay. Liver microsomes were prepared from individual mouse livers as previously described (Paine et al., 1997; Shuster et al., 2014). All steps were carried out at 4°C to minimize protein degradation. Approximately 750 mg of frozen mouse liver tissue was homogenized in 2 ml homogenization buffer (50 mM KPi buffer containing 0.25 M sucrose and 1 mM EDTA) using Omni Bead Ruptor Homogenizer (Omni International, Kennesaw, GA). The homogenate was then centrifuged for 30 minutes at 15,000*g*, and the resulting supernatant was centrifuged for 70 minutes at 120,000*g*. The pellet containing microsomes was washed once with the buffer (10 mM KPi, 0.1 mM KCl, 1 mM EDTA, pH 7.4) before a final centrifugation for 70 minutes at 120,000*g*. The final pellet was resuspended in 1 ml of the storage buffer (50 mM KPi, 0.25 M sucrose, 10 mM EDTA, pH 7.4) and stored in 200 μl aliquots at −80°C until analysis. Microsomal protein concentration was determined by using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

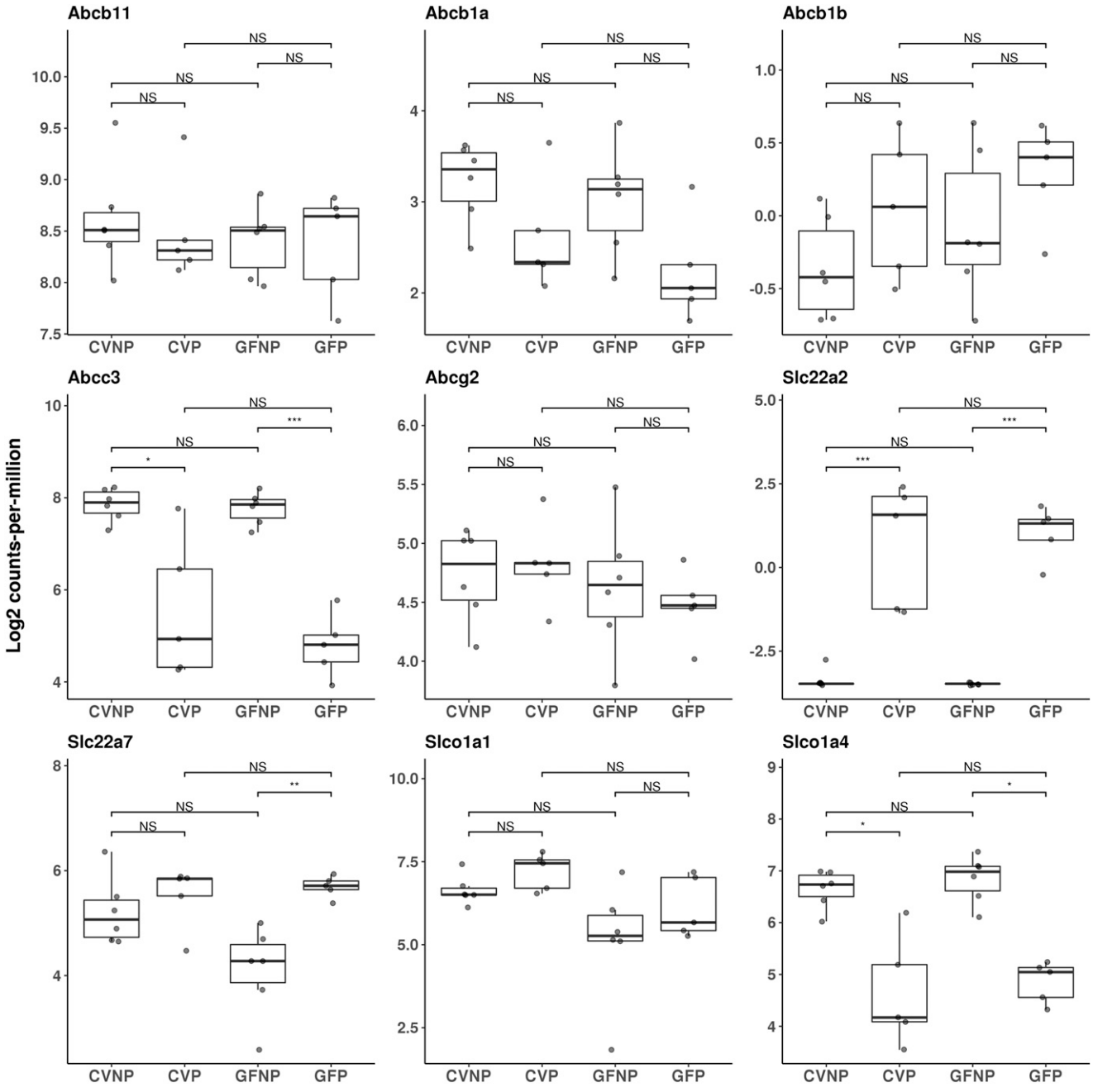

Fig. 4. Effect of pregnancy and microbiome on mRNA expression of hepatic transporters. Shown are boxplots with individual scatters of RNA-seq analysis data of hepatic transporters from female C57BL/6 mice. Data illustrates individual log2 counts per million for each DMET. All pregnant mice used were on gestation day 15. *FDR $<$ 0.1; **FDR $<$ 0.01; ***FDR $<$ 0.001.

CYP3A activities in individual liver microsomal preparations were determined by using the Promega P450-Glo Screening Kit (Promega Corporation, Madison, WI) according to the manufacturer's instruction. Briefly, liver microsomes (7 μg per reaction) from CV and GF mice were preincubated with 3 μM luminogenic P450-Glo Luciferin-IPA for 10 minutes at 37°C. In the case of the inhibition assay, ketoconazole was added to the reaction mixture to a final concentration of 5 μM as previously described (Perloff et al., 1999). Then, NADPH regeneration system was added to the mixture to initiate reaction. Total reaction volume was 75 μl. After incubation for 10 minutes at 37°C, reaction was stopped by the addition of equal volume of the luciferin detection reagent at room temperature, and the mixture sat for 20 minutes. Cyp3a-mediated reaction results in generation of a luciferin product, which was measured by a Glomax 96 Microplate Luminometer (Promega Corporation). Light signal detected reflects the magnitude of Cyp3a activity. Both the substrate concentration and time of incubation were optimized to fall within the linear range of human CYP3A activity, as reported by Promega and described in its instruction. Control incubations using recombinant CYP3A4 microsomes were also performed in parallel as positive control. Incubations were done in triplicates and repeated once. The same assay kit has previously been used to measure mouse Cyp3a activity (Lee et al., 2013; Selwyn et al., 2015; Li et al., 2017).

Quantification of Plasma Bile Acids and Steroid Hormones. Bile acids were extracted from mouse plasma as previously described (Alnouti et al., 2008). Briefly, 50 μl of plasma sample was mixed with 10 μl of internal standard solution (20 μg/ml d4-G-CDCA, 10 μg/ml d4-G-CDCA in 50% MeOH) and vortexed well. Protein was precipitated by adding 500 μl of ice-cold methanol. The mixture was centrifuged at 12,000*g* for 10 minutes at 4°C. The resulting supernatant was kept in a new tube, and the pellet was put through the same steps. The resulting supernatant was combined with the previous one, dried under

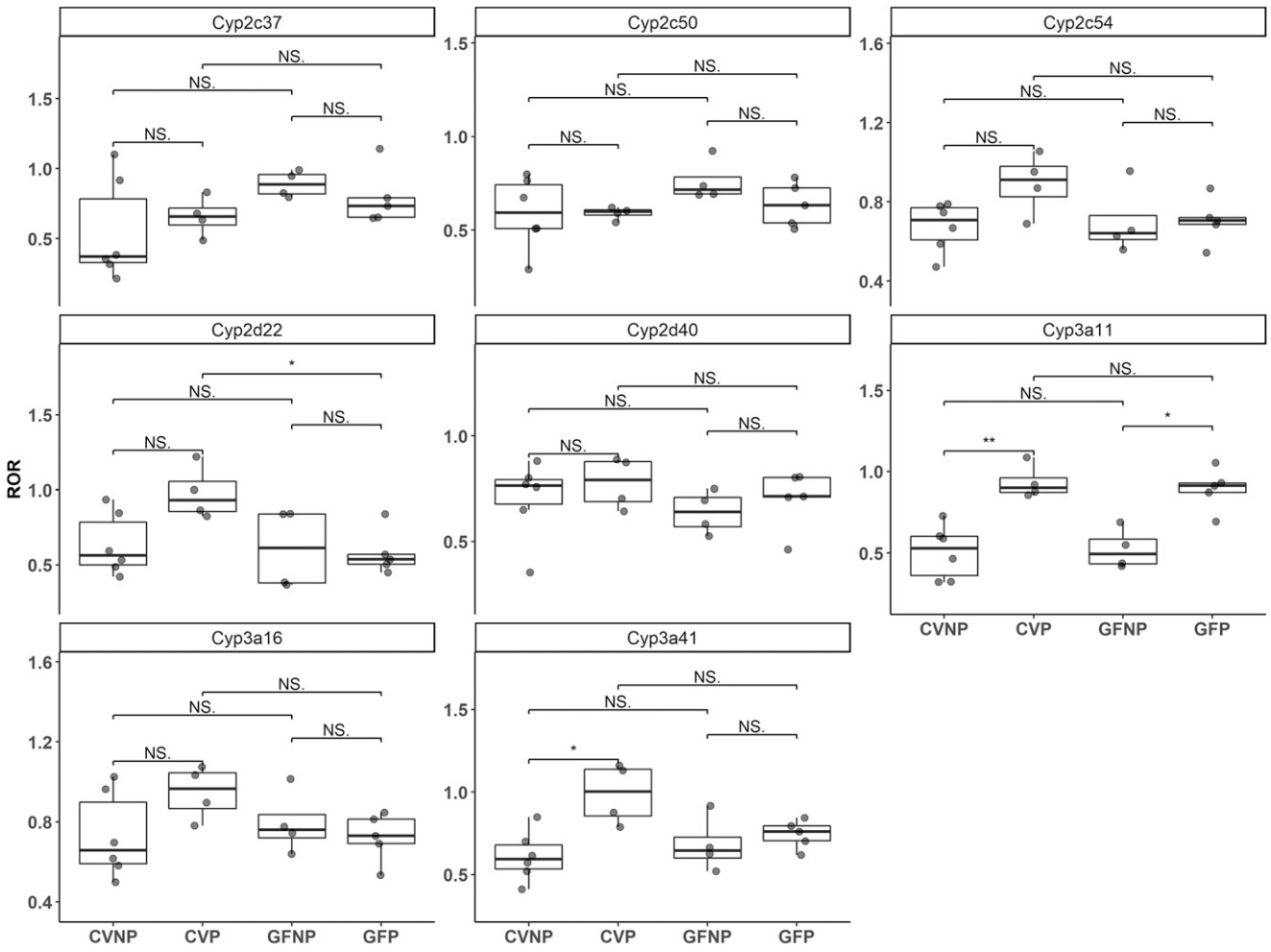

Fig. 5. Effect of pregnancy and microbiome on protein expression of hepatic P450 enzymes. Shown are boxplots with individual data points of relative protein levels of hepatic P450 enzymes in C57BL/6 female mice. Shown are means ± S.E. for selected enzymes or transporters in the liver from four to six mice. Data illustrates ratio-of-ratio estimates for each DMET. All pregnant mice used were on gestation day 15. $*P < 0.05$; $**P < 0.01$ by Wilcoxon signed-rank test.

vacuum (30°C), and reconstitute in 100 μl of 1:1 methanol-water (v/v). The suspension was centrifuged again at 12,000*g* for 10 minutes at 4°C, and 50 μl was subjected to ultraperformance LC-MS/MS for analysis. Chromatographic conditions and instrument settings were the same as previously described (Alnouti et al., 2008) with modifications. Samples were eluted using mobile phases consisting of 20% acetonitrile and 10 mmol/L ammonium acetate in aqueous solutions (A) and 80% acetonitrile and 10 mmol/L ammonium acetate in aqueous solutions (B) at a flow rate of 0.400 ml/min. Five microliters of each sample was injected on column for analysis using negative ionization mode.

Primary bile acids CA, CDCA, ursodeoxycholic acid (UDCA), α/β-murine cholic acid (α/β-MCA), and their respective taurine conjugates (TCA, T-CDCA, TUDCA, T-α/β-MCA) as well as secondary bile acids DCA, hyodeoxycholic acid (HDCA), LCA, murideoxycholic acid (MDCA), ω-muricholic acid (ω-MCA), and their respective taurine conjugates (T-ω-MCA, T-DCA, T-HDCA, and T-LCA) were quantified. Calibrator and different quality control samples were prepared by adding the appropriate amount of the different standard stock solutions and were extracted by using the similar sample preparation procedure described above.

Steroid hormones were extracted from mouse plasma as previously described (Basit et al., 2018). Briefly, 200 μl of methanol containing an internal standard cocktail (10 μg/ml estrone-d4, 10 μg/ml cortisol-d4, 10 μg/ml 11-deoxycortisol-d5, 10 μg/ml Dehydroepiandrosterone-d5 (DHEA-d5), 10 μg/ml progesterone-d9, 10 μg/ml 17OH-progesterone-d8, and 10 μg/ml 17OH-pregnenolone-d3) was added to 50 μl of plasma samples, and the mixture was mixed vigorously for 1 minute followed by a centrifugation at 3500*g* for 10 minutes at 4°C. The supernatant was moved to a new microcentrifuge tube and dried down under N_2. Samples were reconstituted in 50% methanol and analyzed by LC-MS/MS, using the same instrument and conditions as previously described (Basit et al., 2018).

Statistical Analyses for Plasma Metabolite Quantification and Microsome Activity. Data are presented as means ± S.E. (or S.D.) of independent samples from four to six different mice. Statistical significance of the difference between two groups was determined by the Wilcoxon *t* test, with $P < 0.05$ to be considered as significant. Data analyses were performed using R (v3.5.1 and v3.6.1) and GraphPad Prism (GraphPad Prism 5.01, La Jolla, CA).

Results

Validation of RNA-Seq Data by qRT-PCR. A direct comparison of the two approaches (RNA-seq and qRT-PCR) is shown in Fig. 1 for six selected genes (*Cyp3a16*, *Cyp2b13*, *Cyp2c50*, *Sult4a1*, *Abcc3*, and *Slco1a4*). Overall, gene expression levels determined by the two approaches showed the same pattern of pregnancy-induced changes in both CV and GF mice (upregulation or downregulation). More specifically, *Cyp2b13*, *Cyp2c50*, *Sult4a1*, *Abcc3*, and *Slco1a4* demonstrated downregulation by both RNA-seq and qRT-PCR analyses. As for *Cyp3a16,* downregulation in GFNP group and upregulation in CVNP, CVP, and GFP groups were observed. The consistency of these results established the accuracy and reliability of the more comprehensive RNA-seq analysis.

RNA-Seq Analysis of Hepatic DMETs. To determine whether pregnancy and the microbiome interact to affect the expression of host

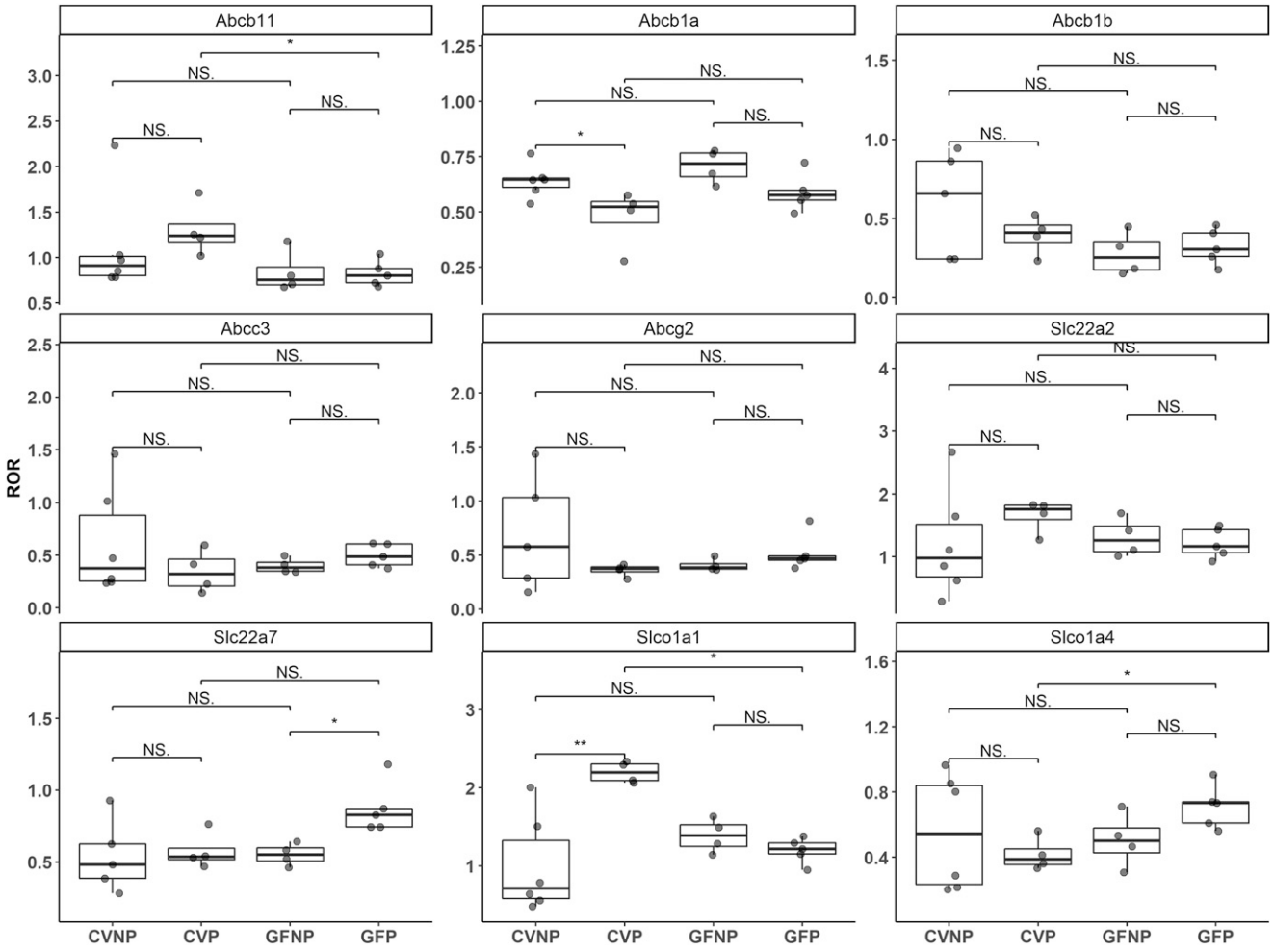

Fig. 6. Effect of pregnancy and microbiome on protein expression of hepatic transporters. Shown are boxplots with individual data points of relative protein levels of hepatic transporters in C57BL/6 female mice. Shown are means ± S.E. for selected enzymes or transporters in the liver from four to six mice. Data illustrates ratio-of-ratio estimates for each DMET. All pregnant mice used were on gestation day 15. $*P < 0.05$; $**P < 0.01$ by Wilcoxon signed-rank test.

hepatic DMETs, we performed RNA-seq analysis of liver tissues from CVNP ($n = 6$), CVP ($n = 5$), GFNP ($n = 6$), and GFP ($n = 5$) C57BL/6 mice. Specifically, we examined the mRNA expression profiles of genes important for xenobiotic, bile acid, and steroid hormone disposition, including phase I enzymes (P450 enzymes), phase II enzymes [UDP-glucuronosyltransferases (*Ugt*), sulfotransferases (*Sult*), and glutathione *S*-transferases (*Gst*)], and transporters [ATP-binding cassette (*Abc*) transporters, solute carrier (*Slc*) transporters, and solute carrier organic anion (*Slco*) transporters]. Differential changes in the expression of key DMETs by pregnancy in CV and GF mice are shown in Table 1. A complete list of DMETs as determined by RNA-seq analysis can be found in Supplemental Table 2.

There were no significant differences in the expression of DMETs between CVNP and GFNP mice, with either no change or slight trend of downregulation for most of the DMETs listed in Table 1 in GFNP mice compared with CVNP mice. Comparing pregnant mice, we found the mRNA levels of two genes, *Cyp2b13* and *Cyp2c38*, were higher, 5.3-fold (FDR < 0.001) and threefold (FDR = 0.013), respectively, in GFP mice compared with CVP mice (Table 1). The expression of other DMET genes was not significantly different between CVP and GFP mice (Table 1).

In both CV and GF mice, pregnancy induced significant changes in the expression of many DMETs in phase I (Fig. 2) and phase II (Fig. 3) enzymes as well as transporters (Fig. 4) relative to nonpregnant mice. Pregnancy altered the expression of DMETs with an overall similar trend but with different magnitudes of changes in CV and GF mice. For example, pregnancy decreased the mRNA expression of *Cyp2c37*, *Cyp2c38*, *Cyp2c50*, and *Cyp2c54* by 70% (FDR < 0.01), 60% (FDR < 0.01), 80% (FDR < 0.001), and 60% (FDR < 0.01), respectively, in CV mice, whereas in GF mice, no significant or much smaller changes by pregnancy were observed for the same genes (Table 1). Similarly, *Cyp2d40* was induced 3.9-fold (FDR < 0.001) and 5.4-fold (FDR < 0.001) by pregnancy, respectively, in CV and GF mice (Fig. 2; Table 1). Among all the P450 isoforms analyzed, CV and GF mice showed the most differences in the gene expressions of *Cyp3a* isoforms in response to pregnancy (Fig. 2; Table 1). We observed a 70% downregulation of *Cyp3a11* by pregnancy in GF mice (FDR = 0.005) but a nonsignificant downregulation in CV mice by pregnancy. The mRNA levels of *Cyp3a16* were induced 20.6-fold (FDR < 0.001) by pregnancy in CV mice versus 128-fold (FDR < 0.001) in GF mice. Likewise, *Cyp3a41a* and *Cyp3a41b* were induced by pregnancy 4.8-fold and 74.2-fold (FDR < 0.05 and FDR < 0.001, respectively) in CV mice compared with 10.8-fold and 181.7-fold (FDR = 0.002 and FDR < 0.001, respectively) in GF mice. *Cyp3a44* was upregulated by 14.3-fold (FDR < 0.001) by pregnancy in CV mice versus 30.5-fold (FDR < 0.001) in GF mice.

Of all phase II enzymes that were differentially expressed during pregnancy, we noted that the most significant changes induced by

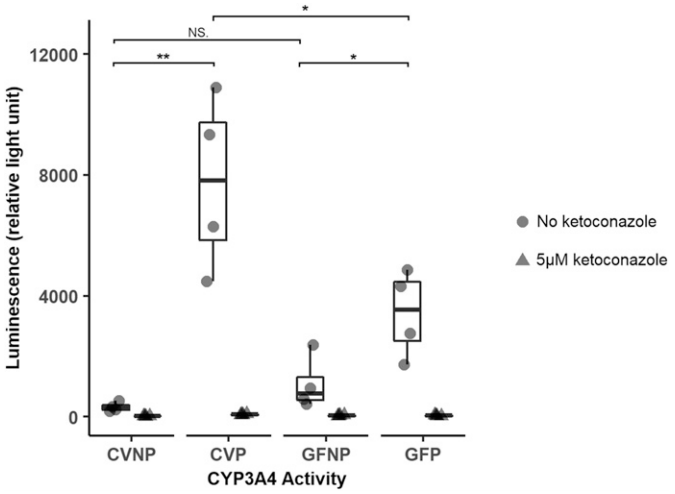

Fig. 7. Effect of pregnancy and microbiome on hepatic Cyp3a activity. Shown are boxplots of individual data points of hepatic Cyp3a activity, which is presented as relative light unit. Circles represent incubations without an inhibitor, and triangles indicate incubations in the presence of the inhibitor ketoconazole at 5 μM. Data shown are means ± S.E. of hepatic Cyp3a activity in liver microsomes isolated from four to six mice from one representative experiment. The experiment was done in triplicate and repeated, and similar results were obtained. All pregnant mice used were on gestation day 15. $*P < 0.05$; $**P < 0.01$ by Wilcoxon signed-rank test.

pregnancy were associated with *Sult3a2* and *Sult4a1* in both CV and GF mice. We observed a 53.9-fold (FDR < 0.001) induction by pregnancy in CV mice versus a 226.7-fold (FDR < 0.001) induction in GF mice for *Sult3a2* and a 287.7-fold (FDR < 0.001) induction in CV mice versus only a nonsignificant induction in GF mice for *Sult4a1* (Fig. 3; Table 1).

For transporters, *Abcc3* and *Slco1a4* were downregulated by pregnancy to a similar extent in CV and GF mice (Fig. 4; Table 1). *Abcc3* was downregulated by pregnancy by 70% (FDR < 0.05) and 90% (FDR < 0.001), respectively, in CV and GF mice. *Slco1a4* was decreased 60% by pregnancy in CV mice (FDR < 0.05) but nonsignificantly in GF mice. Pregnancy induced *Slc22a2* and *Slc22a7* by 561.5-fold (FDR < 0.001) and 3.1-fold (FDR = 0.004), respectively, in GF mice versus 260.5-fold (FDR < 0.001) induction of *Slc22a2* and a nonsignificant induction of *Slc22a7* in CV mice (Fig. 4; Table 1).

Quantification of Relative Protein Abundance of Selected Hepatic DMETs by Targeted Proteomics. To investigate and compare how protein levels of hepatic DMETs were changed by pregnancy in CV and GF mice, we performed quantitative proteomics of selected hepatic DMETs in liver tissues. Because of the limited amount of isolated liver membrane proteins available, we performed targeted proteomics only on a set of selected DMETs. Selection of DMETs was based on a priori knowledge of their importance in overall xenobiotic disposition (Nelson et al., 2004). Relative protein abundance of these DMETs was quantified using LC-MS/MS–based proteomics, and results are shown in Figs. 5 and 6.

Of the phase I enzymes, we opted to quantify mouse isoforms homologous to human CYP2C19 (CYP2C37, CYP2C50, and CYP2C54), human CYP2D6 (CYP2D22 and CYP2D40), and human CYP3A4 (CYP3A11, CYP3A16, CYP3A41A, and CYP3A41B). Because there are no distinct methods to separate CYP3A41A and CYP3A41B, the results for these two enzymes were combined as CYP3A41. Overall, the effects of pregnancy on protein expression profiles of these P450 enzymes in CV and GF mice were notably different from the effects of pregnancy on respective mRNA expression profiles. Specifically, we found that pregnancy significantly increased the protein levels of CYP3A11 approximately twofold in both CV and GF mice (Fig. 5), whereas the mRNA levels of *Cyp3a11* were downregulated by pregnancy in CV and GF mice (Fig. 2). There were no significant differences in the protein levels of CYP3A16 between CVNP and CVP or between GFNP and GFP; however, the protein level of CYP3A16 in CVP mice was significantly higher (~25%) than in GFP mice (0.95 ± 0.04 vs. 0.72 ± 0.03, $P < 0.05$) (Fig. 5). The effect of pregnancy on protein expression of CYP3A41 was similar to its effect on mRNA expression with a trend of higher abundance in pregnancy for both CV and GF mice; however, a statistically significant increase by pregnancy was observed only in CV mice (Fig. 5). No significant differences in the protein levels of CYP2C and CYP2D isoforms between CVNP and CVP or between GFNP and GFP were observed (Fig. 5). Like CYP3A16, the protein levels of CYP2D22 in CVP mice were significantly higher by ~40% than those in GFP mice (0.98 ± 0.03 vs. 0.58 ± 0.02, $P < 0.05$) (Fig. 5).

We also quantified various transporters because of their importance in drug disposition, including ABCB11 (bile salt export pump), ABCB1A/B (P-glycoprotein), ABCC3 (Mrp3), ABCG2 (breast cancer resistance protein), SLC22A2 (organic cation transporter 2), SLC22A7 [organic anion transporter (Oat) 2], SLCO1A1 (Oatp1a1), and SLCO1A4 (Oatp1a4). Similar to CYP3A16 and CYP2D22, there were no significant differences in the protein levels of ABCB11 between CVNP and CVP or between GFNP and GFP; however, the protein levels of ABCB11 in CVP mice were significantly greater (~37%) than those in GFP mice (1.3 ± 0.07 vs. 0.82 ± 0.03, $P < 0.05$) (Fig. 6). We also observed significantly decreased protein abundance of SLCO1A1 in GFP mice compared with CVP mice (1.20 ± 0.03 vs. 2.20 ± 0.03, $P < 0.001$). The protein levels of SLCO1A4 in GFP mice were significantly higher than those in CVP mice (0.71 ± 0.03 vs. 0.42 ± 0.03, $P < 0.05$), but there were no significant differences between GFNP and CVNP mice (Fig. 6). Pregnancy significantly increased the protein levels of SLC22A7 ~60% in GF mice (0.87 ± 0.04 vs. 0.55 ± 0.02, $P < 0.05$) but not in CV mice. No significant changes were observed in the abundance of ABCB1A/B, ABCC3, and ABCG2 proteins because of pregnancy or the microbiome.

Activity of Hepatic Cyp3a Enzymes Determined by Liver Microsomal Incubations. To confirm pregnancy-induced changes in protein expression of Cyp3a enzymes, microsomal incubations were performed to determine Cyp3a activity. We used a luciferin CYP3A probe to measure the amount of metabolite formed at a single incubation time point, which all fell within the linear range of product formation. Liver microsomes from CVP mice exhibited ~30-fold higher CYP3A activities ($P < 0.05$) than those from CVNP mice, whereas liver microsomes from GFP mice showed only a ~threefold induction ($P < 0.05$) of CYP3A activity compared with GFNP mice (Fig. 7). Furthermore, the addition of ketoconazole (a potent known CYP3A inhibitor) at 5 μM completely abolished metabolite formation with microsomes from both CV and GF mice (Fig. 7), suggesting that the enzymatic activity was due to CYP3A enzymes. Blank control and human recombinant CYP3A4 controls were also assayed in parallel to verify assay functionality (data not shown).

Quantification of Plasma Bile Acids and Steroid Hormones. Because secondary bile acids generated by the gut microbiome may play an important role in regulating the expression of certain DMETs including Cyp3a enzymes (Nelson et al., 2004; Chu et al., 2013), we quantified plasma concentrations of primary and secondary bile acids in all mice by LC-MS/MS.

The plasma concentrations of the primary bile acids, α-MCA and β-MCA, in GFNP and GFP mice were lower than those in CVNP and CVP mice (Fig. 8). Pregnancy significantly increased the plasma concentrations of β-MCA in CV mice but had no effect in GF mice. The microbiome had the opposite effect on taurine conjugates, with higher plasma concentrations of T-α-MCA and T-β-MCA in GF mice

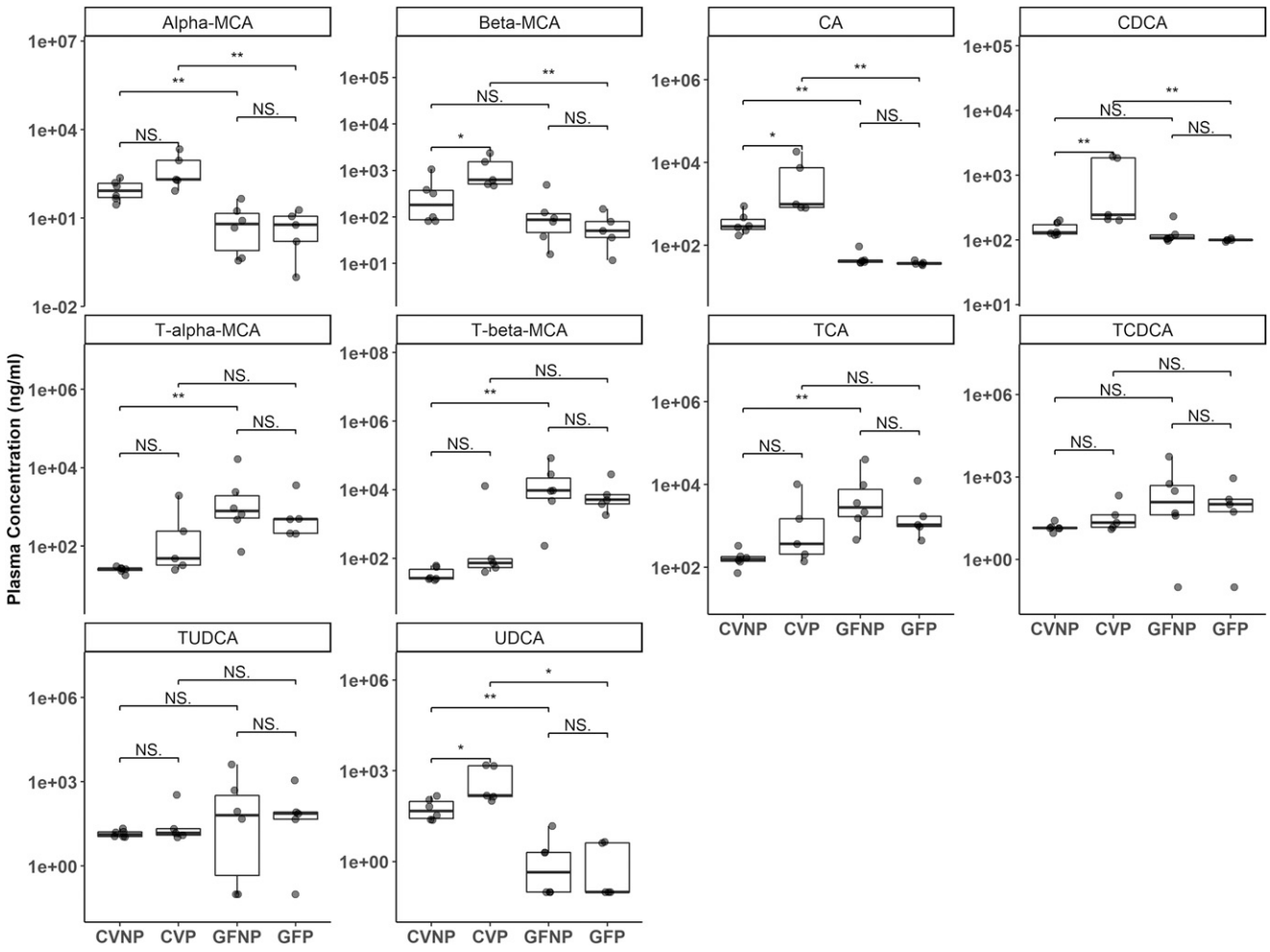

Fig. 8. Effect of pregnancy and microbiome on plasma concentrations of primary bile acids. Shown are boxplots with individual data points of plasma concentrations of primary bile acids in C57BL/6 female mice. Concentrations were determined using targeted LC-MS/MS quantification and reported as nanograms per milliliter. Shown are means ± S.E. for selected bile acids or steroid hormones in the plasma from five to six mice. All pregnant mice used were on gestation day 15. $^{*}P < 0.05$; $^{**}P < 0.01$ by Wilcoxon signed-rank test.

than in CV mice, regardless of pregnancy. The plasma concentrations of CA were increased ~14-fold by pregnancy in CV mice with no significant changes between GFP and GFNP mice. Moreover, the plasma concentrations of CA in GF mice were significantly lower than those in CV mice regardless of pregnancy (Fig. 8). The pattern of plasma concentrations of UDCA in all mice was similar to CA. Neither pregnancy nor the microbiome significantly affected the plasma concentrations of taurine conjugates of CA, CDCA, and UDCA, with the exception that the plasma concentrations of T-CA in GFNP mice were 55-times greater than those in CVNP mice (Fig. 8).

As expected, the plasma concentrations of most of the secondary bile acids were lower in GF mice versus CV mice, likely because of the lack of gut bacteria to synthesize them from primary bile acids (Fig. 9; Supplemental Table 3). The plasma concentrations of DCA, HDCA, LCA, ω-MCA, T-ω-MCA, T-DCA, and T-LCA in GF mice were all significantly decreased versus those in CV mice, regardless of pregnancy (Fig. 9). Interestingly, in addition to a decrease in plasma concentrations of LCA in GFNP mice compared with CVNP mice (~11-fold decrease, $P < 0.05$), there was a further decrease of ~fivefold by pregnancy from GFNP to GFP mice ($P < 0.05$). Pregnancy also seemed to affect the production of some secondary bile acids. For example, pregnancy increased the plasma concentration of DCA ~sevenfold ($P < 0.05$) in CV mice but had no effect in GF mice. Pregnancy also significantly increased the plasma concentrations of HDCA in CV mice (ninefold, $P < 0.05$) but caused a fourfold reduction ($P < 0.05$) in GF mice. Neither pregnancy nor the microbiome significantly affected the plasma concentrations of T-HDCA (Fig. 9). With respect to MDCA, pregnancy significantly increased its plasma concentrations only in CV mice but not in GF mice (Fig. 9). As expected, the plasma concentrations of MDCA were decreased in GF mice versus CV mice regardless of pregnancy, but the changes seemed to be small (Fig. 9; Supplemental Table 3).

Pregnancy-related steroid hormones have been shown to regulate the expression of DMETs. Therefore, we also determined plasma concentrations of steroid hormones in all mice. As expected, pregnancy significantly increased the plasma concentrations of various steroid hormones, including 11-deoxycorticosterone, 17-OH-pregnenolone, 17-OH-progesterone, corticosterone, cortisol, and progesterone, 38-fold to 125-fold in both CV and GF mice (Fig. 10; Supplemental Table 4). The lack of the microbiome did not seem to alter pregnancy-induced levels of these hormones. The plasma concentrations of aldosterone and cortisone were significantly elevated by pregnancy in GF mice but not in CV mice (Fig. 10). The plasma concentrations of estradiol were slightly but significantly decreased by pregnancy in CV mice but not in GF mice. The lack of the microbiome did not affect the plasma concentrations of estradiol in both pregnant and nonpregnant mice (Fig. 10). On the other

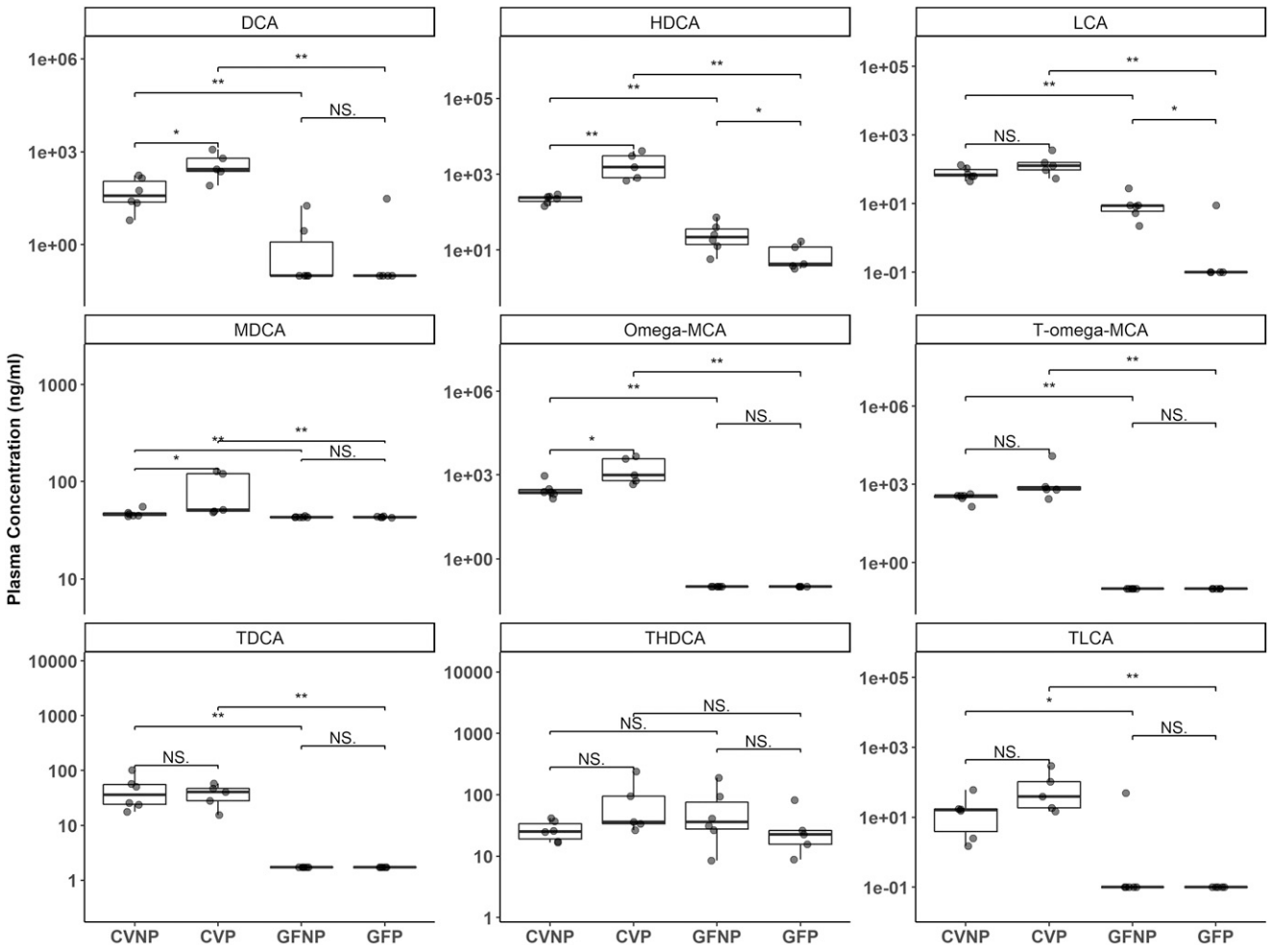

Fig. 9. Effect of pregnancy and microbiome on plasma concentrations of secondary bile acids. Shown are boxplots with individual data points of plasma concentrations of secondary bile acids in C57BL/6 female mice. Concentrations were determined using targeted LC-MS/MS quantification and reported as nanograms per milliliter. Shown are means ± S.E. for selected bile acids or steroid hormones in the plasma from five to six mice. All pregnant mice used were on gestation day 15. $*P < 0.05$; $**P < 0.01$; by Wilcoxon signed-rank test.

hand, we did not observe significant effects of pregnancy or the microbiome on pregnenolone, estrone, and Dehydroepiandrosterone (DHEA).

Discussion

In this study, we investigated the combined effects of pregnancy and microbiome on hepatic DMETs. This study used the same C57BL/6 strain as previous studies that investigated the effects of microbiome on hepatic DMETs in male mice (Selwyn et al., 2015, 2016). Both this and the previous studies showed a downward trend in mRNA of *Cyp3a* isoforms in GF versus CV mice; however, the magnitude of down-regulation observed in this study was much less pronounced. This could be due to sex difference. The previous studies also reported a significant induction of *Cyp4a* genes by the microbiome knockout, whereas we did not. Again, this could be due to *Cyp4a* genes, which are sex-divergent (Renaud et al., 2011). Regulation of *Cyp4a14* was strongly influenced by androgen (Zhang and Klaassen, 2013), which is expectedly low in our female mice. Other factors that are known to control the gut microbiome ecosystems (e.g., age of mice, diet, and housing conditions) and might be different between this and previous studies could also potentially contribute to the discrepancy.

We previously showed that pregnancy alters hepatic DMETs in conventional FVB mice (Shuster et al., 2013). Overall, our results are in good agreement with our previous studies. For example, we reported that *Cyp3a11* mRNA was decreased, whereas mRNA of *Cyp3a16* and *Cyp3a41* was increased during pregnancy. Consistently, this study also showed downregulation and induction of *Cyp3a11* and *Cyp3a16/Cyp3a41*, respectively, by pregnancy in CV mice. We previously reported significant downregulation of *Cyp2c* genes and *Mrp3/Abcc3* by pregnancy similar to this study in CV mice (Shuster et al., 2013). There are some inconsistencies in the magnitude of pregnancy-induced change for some DMETs between this and our previous studies. For example, this study showed a 20.6-fold induction of *Cyp3a16* by pregnancy, whereas our previous studies reported an induction of only 60% on the same gd 15 (Shuster et al., 2013). Our previous studies used FVB mice. The two different mouse strains are known to possess significantly different basal microbiota (Ahn et al., 2018). Thus, the difference in mouse strain may contribute to the different magnitude of pregnancy-induced change for some DMETs between this and our previous studies.

The *Cyp3a* isoforms (*Cyp3a11*, *Cyp3a16*, *Cyp3a41*, and *Cyp3a44*) were of particular interest because of the prominent role of their human CYP3A4 homolog in drug metabolism. We therefore systematically examined their mRNA and protein expression along with catalytic activity. We found that *Cyp3a11* mRNA was significantly decreased by pregnancy in both CV and GF mice but with a much greater magnitude of downregulation in GF versus CV mice. Downregulation of *Cyp3a11*

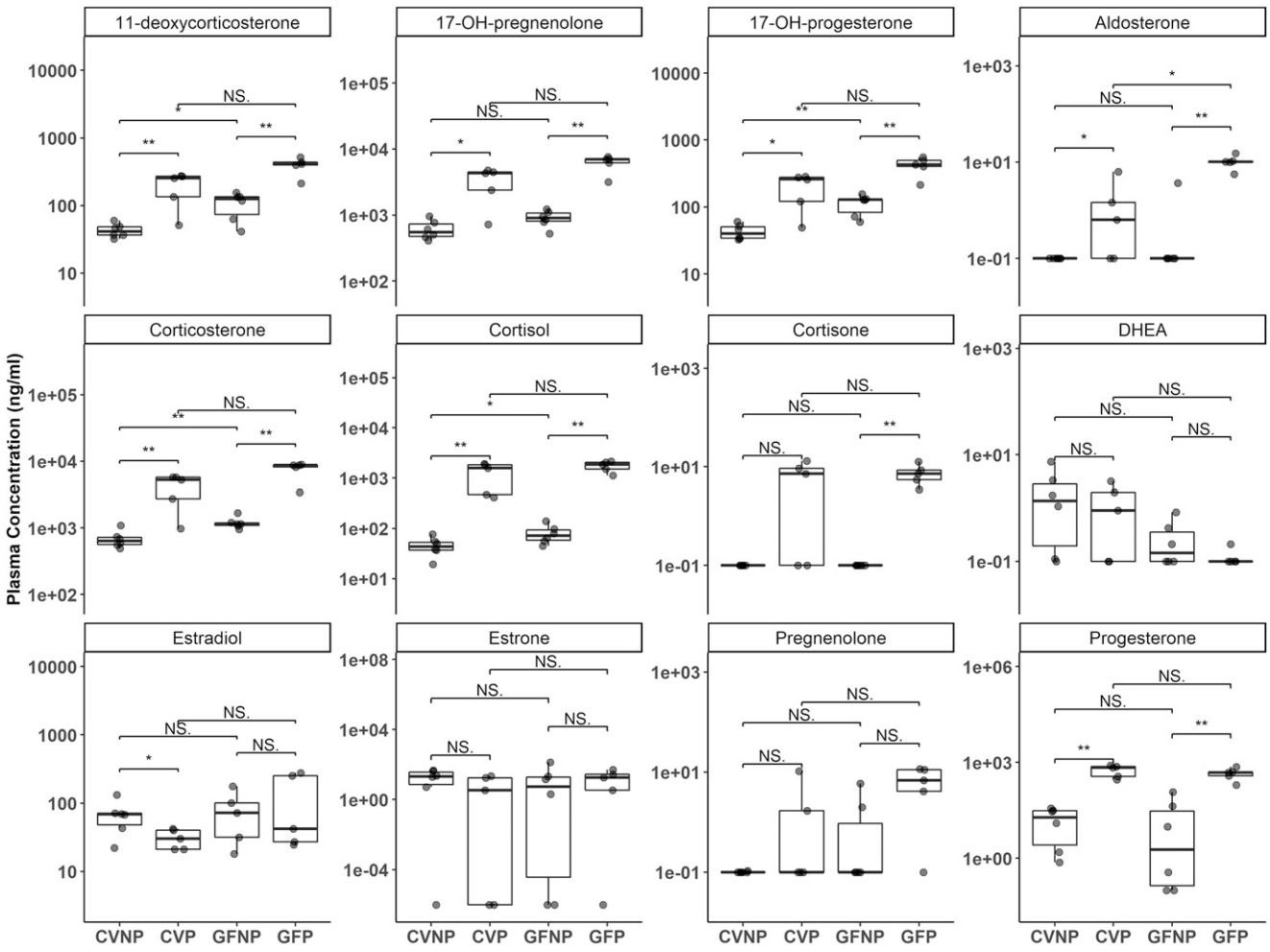

Fig. 10. Effect of pregnancy and microbiome on plasma concentrations of steroid hormones. Shown are boxplots with individual data points of plasma concentrations of steroid hormones in C57BL/6 female mice. Concentrations were determined using targeted LC-MS/MS quantification and reported as nanograms per milliliter. Shown are means ± S.E. for selected bile acids or steroid hormones in the plasma from five to six mice. All pregnant mice used were on gestation day 15. $*P < 0.05$; $**P < 0.01$ by Wilcoxon signed-rank test.

mRNA by pregnancy has been reported in our previous studies in CV mice, but the mechanistic reasoning is unknown (Zhang et al., 2008; Shuster et al., 2013). The greater magnitude of down-regulation of *Cyp3a11* mRNA by pregnancy in GF mice could be due to the combined effects of the lack of secondary bile acids, which would otherwise compensate for the decreased *Cyp3a11* gene expression during pregnancy in CV mice. Yet, targeted proteomics revealed an opposite effect of pregnancy on the protein levels of CYP3A11, with a comparable twofold induction in both CV and GF mice. In contrast to *Cyp3a11,* the mRNA levels of all other *Cyp3a* isoforms were induced by pregnancy in both CV and GF mice. Although the mRNA levels of these *Cyp3a* isoforms in CVP and GFP mice were comparable, the fold-induction in GF mice was generally much greater than that in CV mice (e.g., 128-fold in GF mice vs. 20.6-fold in CV mice for *Cyp3a16*) (Table 1). This was due to the lower baseline levels of these *Cyp3a* genes in GFNP mice compared with CVNP mice, which were likely caused by the lack of secondary bile acids. Surprisingly, pregnancy induced the protein levels of CYP3A16 and CYP3A41 in CV mice but had limited effect in GF mice. Although circadian patterns of P450 mRNA transcripts have been reported (Zhang et al., 2009), all mice were sacrificed around the same time of day, and thus the contribution of time of tissue collection is unlikely to be the source of changes observed. Next, we examined the effects of pregnancy and microbiome on the overall CYP3A activity. At present, it is not possible to determine activities of individual CYP3A isoforms because of the lack of specific probes or selective inhibitors for individual mouse CYP3A isoforms. Thus, the CYP3A activities we measured reflected the overall combined activities of all CYP3A isoforms. The CYP3A activities were significantly induced by pregnancy in both CV and GF mice but with a much greater induction in CV mice versus GF mice. The activity data agreed with the overall trend of pregnancy-induced increase in CYP3A protein and are also consistent with our previous studies showing that the mouse CYP3A activities for testosterone and glyburide were significantly induced by pregnancy (Zhang et al., 2008; Shuster et al., 2014). However, for the first time, we showed that the microbiome can significantly alter the magnitude of pregnancy induction of CYP3A activity. The lower pregnancy induction of CYP3A activity in GF mice could reflect only the activities of the CYP3A isoforms other than CYP3A11 that were not induced at the protein level (Fig. 5). Future studies are needed to explore the mechanisms of microbiome-induced changes in CYP3A activity.

There was a disconnection between the mRNA and protein expression data for many DMETs in addition to *Cyp3a11* as described above. For example, pregnancy drastically decreased the mRNA levels of *Cyp2c50* and *Cyp2c54* but had no significant impact on their protein levels in both CV and GF mice. Likewise, the mRNA levels of *Abcc3* were

significantly decreased by pregnancy in CV and GF mice, but its protein levels were not affected by pregnancy at all. These inconsistencies could be due to the relatively small sample size and large variations in analysis (e.g., proteomics) for certain DMETs, yet the data for CYP3A isoforms seem to be reliable because both the mRNA and/or activity data for the CYP3A isoforms are fully consistent with previous studies. The discrepancy between mRNA and protein expression is not uncommon and could be due to post-transcriptional or epigenomic regulation of gene expression, as shown in numerous studies (Martínez-Jiménez et al., 2007; Takagi et al., 2008; Smutny et al., 2013; Wang et al., 2019). Recent studies showed that the gut bacteria *Akkermansia muciniphila* can affect the concentration of N6-methyladenosine in the intestines and the liver, and N6-methyladenosine methylation can greatly alter RNA transcription processes, leading to epitranscriptomic changes that may explain the gap between mRNA and protein data (Zhao et al., 2017; Miro-Blanch and Yanes, 2019). MicroRNA (miRNA) modifiers can also alter the transcript-protein relationship. A recent study reported bacteria phylum such as *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* have significant effects on miRNA expression in humans (Yuan et al., 2018). Changes in miRNA expression because of bacteria shift or the microbiome knockout could also lead to changes in downstream gene regulation and result in variations in gene translation. Future studies are needed to explore the mechanisms of microbiome-induced disconnection between mRNA and protein expression of DMETs.

Cortisol has been shown to induce the expression of CYP3A through activation of glucocorticoid receptor, which in turn induces PXR, and then PXR induces transcription of CYP3A expression (Sachar et al., 2019). This could be the potential mechanism by which hepatic CYP3A is induced during pregnancy. Supporting this hypothesis, the plasma concentrations of cortisol were markedly increased by pregnancy in both CV and GF mice, and the mRNA levels of all *Cyp3a* isoforms but *Cyp3a11* were induced by pregnancy. Secondary bile acids have been shown to be PXR ligands that can induce CYP3A (Dempsey et al., 2019). This explains our findings that the baseline mRNA levels of all *Cyp3a* isoforms in GFNP mice were generally lower than those in CVNP mice. However, the absence of secondary bile acids did not affect the induction of *Cyp3a16* and *Cyp3a41* mRNA by pregnancy, which is consistent with the finding that pregnancy-induced hormone production was generally not affected by the microbiome knockout. This suggests that the lack of microbiome does not interfere with pregnancy-mediated induction of *Cyp3a* gene transcription, which is mediated by increased production of steroid hormones during pregnancy. However, the microbiome can alter the magnitude of induction by changing the baseline expression of *Cyp3a* genes (Fig. 2) or influencing pregnancy-induced Cyp3a protein expression (Fig. 5) and, hence, activity (Fig. 7).

In summary, we have shown, for the first time, that the microbiome can affect hepatic DMETs in pregnant mice by altering the magnitude of pregnancy-induced fold-change in the expression (mRNA or protein) and/or the activity of hepatic DMETs. Caution should be taken to translate the data to drug disposition in vivo because of disconnection between mRNA and protein, relatively small sample size, and large variation in analysis of certain DMETs. Nevertheless, these results provide the basis for further mechanistic investigation of microbiome-mediated changes in hepatic DMETs and drug disposition during pregnancy. Future studies should also examine the impact of microbiome on DMETs in other organs of pregnant mice important for drug disposition, including the small intestine.

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Author Contributions

Participated in research design: Han, Wang, Shi, Bammler, Cui, Mao.

Conducted experiments: Han, Pershutkina.

Contributed new reagents or analytic tools: Han, Wang, Shi, Dempsey, Dutta.

Performed data analysis: Han, Wang, Shi, Dempsey, Dutta.

Wrote or contributed to the writing of the manuscript: Han, Wang, Shi, Dempsey, Dutta, Bammler, Cui, Mao.

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Drug Metabolism and Disposition

Impact of Microbiome on Hepatic Metabolizing Enzymes and Transporters in Mice during Pregnancy

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Supplemental Table S1. Surrogate peptides of cytochrome P450s and transporters and their MS/MS parameters for detection

Protein Name	Surrogate Peptide	Peptide Type	Parent Ion (m/z)	Fragment Ion (m/z)
Cyp2c37	DICQSFTNLSK	Light	656.8	796.4
Cyp2c37	DICQSFTNLSK	Light	656.8	709.4
Cyp2c50	YAILLLLK	Light	473.8	712.5
Cyp2c50	YAILLLLK	Light	473.8	599.4
Cyp2c54	ATNGMGIGFSNGSVWK	Light	813.4	1151.6
Cyp2c54	ATNGMGIGFSNGSVWK	Light	813.4	1094.6
Cyp2c54	ESLDVTIPR	Light	343.9	407.2
Cyp2c54	ESLDVTIPR	Light	343.9	350.7
Cyp2d22	MPYTNAVIHEVQR	Light	390.2	476.3
Cyp2d22	MPYTNAVIHEVQR	Light	390.2	476.2
Cyp2d40	GNPESSFNEANLR	Light	717.8	950.5
Cyp2d40	GNPESSFNEANLR	Light	717.8	863.4
Cyp3a11	ALLSPTFTSGK	Light	561.3	937.5
Cyp3a11	ALLSPTFTSGK	Light	561.3	824.4
Cyp3a11	ALLSPTFTSGK	Light	561.3	737.4
Cyp3a16	QDFFPVGIMSK	Light	423.5	731.4
Cyp3a16	QDFFPVGIMSK	Light	423.5	366.2
Cyp3a41	VDFLQLMMNAHNNSK	Light	441.2	599.3
Cyp3a41	VDFLQLMMNAHNNSK	Light	441.2	516.6
Cyp3a41	LQEEIDETLPNK	Light	476.9	701.4
Cyp3a41	LQEEIDETLPNK	Light	476.9	572.3
Cyp3a41	LQEEIDETLPNK	Light	476.9	120.1
BSA	LVNELTEFAK	Light	582.3	708.4
BSA	LVNELTEFAK	Light	582.3	951.5
BSA	AEFVEVTK	Light	461.7	722.4
BSA	AEFVEVTK	Light	461.7	347.2
BSA	AEFVEVTK	Heavy	465.8	859.5
BSA	AEFVEVTK	Heavy	465.8	730.4
BSA	AEFVEVTK	Heavy	465.8	583.4
BSA	AEFVEVTK	Heavy	465.8	484.3
Abcc3	HIFDQVIGPEGVLAGK	Light	840.5	1429.8
Abcc3	HIFDQVIGPEGVLAGK	Light	840.5	1282.7

Abcc3	HIFDQVIGPEGVLAGK	Heavy	844.5	1437.8
Abcc3	HIFDQVIGPEGVLAGK	Heavy	844.5	1290.7
Abcc3	HIFDQVIGPEGVLAGK	Heavy	844.5	1175.7
Abcc3	HIFDQVIGPEGVLAGK	Heavy	844.5	948.6
Abcc3	HIFDQVIGPEGVLAGK	Heavy	844.5	778.5
Abcb11	LSTALSGLLLGFYR	Light	504.3	655.4
Abcb11	LSTALSGLLLGFYR	Light	504.3	699.3
Abcb11	LSTALSGLLLGFYR	Light	504.3	605.4
Abcb11	FYDPCEGMVTLDGHDIR	Light	507.0	825.4
Abcb11	FYDPCEGMVTLDGHDIR	Light	507.0	712.3
Abcb11	FYDPCEGMVTLDGHDIR	Light	507.0	597.3
Abcg2	GEKPVIENLSEFYINSAIYGETK	Light	867.8	982.5
Abcg2	GEKPVIENLSEFYINSAIYGETK	Light	867.8	989.5
Mdr1a	ATVSASHIIR	Light	527.8	983.6
Mdr1a	ATVSASHIIR	Light	527.8	783.4
Mdr1b	GIYFSMVQAGAK	Light	636.3	791.4
Mdr1b	GIYFSMVQAGAK	Light	636.3	704.4
Slco1a1	YLEQQYGK	Light	514.8	623.3
Slco1a1	YLEQQYGK	Light	514.8	662.3
Slco1a1	YLEQQYGK	Heavy	518.8	873.5
Slco1a1	YLEQQYGK	Heavy	518.8	631.3
Slco1a1	YLEQQYGK	Heavy	518.8	662.3
Slc22a2	YEVDWNQSTLDCVDPLSSLAANR	Light	885.1	1043.5
Slc22a2	YEVDWNQSTLDCVDPLSSLAANR	Light	885.1	1131.5
Slc22a2	YEVDWNQSTLDCVDPLSSLAANR	Heavy	889.1	1043.5
Slc22a2	YEVDWNQSTLDCVDPLSSLAANR	Heavy	889.1	928.5
Slc22a2	LNPSFLDLVR	Light	587.3	946.5
Slc22a2	LNPSFLDLVR	Light	587.3	473.8
Slc22a2	LNPSFLDLVR	Heavy	592.3	956.5
Slc22a2	LNPSFLDLVR	Heavy	592.3	859.5
Slc22a2	LNPSFLDLVR	Heavy	592.3	772.4
Slc22a2	LNPSFLDLVR	Heavy	592.3	625.3
Slc22a2	LNPSFLDLVR	Heavy	592.3	478.8
Slc22a7	WLLLAATLPCVPGIISIWWVPESAR	Light	950.2	1343.7
Slc22a7	WLLLAATLPCVPGIISIWWVPESAR	Light	950.2	1230.6
Slco1a4	MYDINSFR	Light	349.2	409.2
Slco1a4	MYDINSFR	Light	349.2	457.7
Slco1a4	MYDINSFR	Light	349.2	376.2

Supplemental Table S2. Pregnancy and microbiome alter the mRNA expression of DMETs in female C57BL/6 livers. This list of genes was generated by comparing mRNA expression in groups between CVP and CVNP, GFP and GFNP, GFNP and CVNP, and GFP and CVP.

Gene Symbol	CVP vs. CVNP		GFP vs. GFNP		GFNP vs. CVNP		GFP vs. CVP	
	Fold Change	*FDR*	Fold Change	*FDR*	Fold Change	*FDR*	Fold Change	*FDR*
Cyp11a1	1.0	1.000	0.4	0.505	1.6	1.000	0.6	0.968
Cyp11b1	1.0	1.000	0.4	0.628	2.8	1.000	1.0	1.000
Cyp17a1	1.7	0.280	2.9	0.010	0.9	1.000	1.6	0.704
Cyp1a1	0.4	0.088	0.7	0.570	1.0	1.000	1.8	0.635
Cyp1a2	0.6	0.322	0.8	0.594	1.0	1.000	1.2	0.930
Cyp1b1	1.1	0.959	1.9	0.462	1.0	1.000	1.7	0.859
Cyp20a1	0.9	0.702	0.9	0.738	0.9	1.000	0.9	0.941
Cyp21a1	2.5	0.729	0.7	0.896	2.7	1.000	0.8	1.000
Cyp21a2-ps	0.7	0.797	0.9	0.932	0.8	1.000	1.0	1.000
Cyp24a1	1.0	1.000	1.0	1.000	1.0	1.000	1.0	1.000
Cyp26a1	3.2	0.076	4.2	0.020	0.9	1.000	1.1	0.996
Cyp26b1	0.0	<0.001	0.1	<0.001	1.9	0.982	5.0	0.289
Cyp26c1	0.8	0.920	0.7	0.814	0.8	1.000	0.7	0.969
Cyp27a1	0.5	0.077	0.6	0.121	0.7	1.000	0.8	0.857
Cyp2a12	1.0	0.975	1.3	0.641	1.0	1.000	1.4	0.824
Cyp2a21-ps	1.6	0.693	1.9	0.484	1.1	1.000	1.3	0.965
Cyp2a22	0.7	0.466	0.7	0.531	1.8	0.811	2.0	0.467
Cyp2a4	2.2	0.052	1.5	0.323	0.7	1.000	0.5	0.413
Cyp2a5	0.6	0.381	0.6	0.436	1.1	1.000	1.2	0.985
Cyp2ab1	0.5	0.709	0.6	0.813	1.2	1.000	1.7	0.969
Cyp2b10	1.2	0.828	0.8	0.837	1.0	1.000	0.6	0.840
Cyp2b13	0.1	<0.001	0.3	<0.001	1.2	1.000	5.3	<0.001
Cyp2b9	0.6	0.136	1.0	0.992	0.8	1.000	1.3	0.799
Cyp2c23	0.7	0.504	0.9	0.874	1.1	1.000	1.4	0.768
Cyp2c29	0.7	0.261	0.7	0.241	1.4	0.953	1.4	0.639
Cyp2c37	0.3	0.002	0.6	0.152	1.0	1.000	1.8	0.401
Cyp2c38	0.4	0.007	1.0	0.965	1.3	1.000	3.0	0.013
Cyp2c39	1.0	0.983	2.4	0.022	1.1	1.000	2.7	0.154
Cyp2c40	0.6	0.141	0.9	0.900	0.8	1.000	1.2	0.902
Cyp2c50	0.2	<0.001	0.5	0.001	1.0	1.000	2.0	0.066
Cyp2c54	0.4	0.001	0.8	0.561	1.0	1.000	2.2	0.074
Cyp2c55	0.5	0.216	0.1	<0.001	2.8	0.319	0.8	0.974
Cyp2c67	0.4	0.012	0.6	0.279	1.4	1.000	2.3	0.238
Cyp2c68	0.7	0.527	1.1	0.930	0.9	1.000	1.3	0.841
Cyp2c69	1.0	0.998	2.6	0.020	0.6	0.982	1.6	0.708
Cyp2c70	1.9	0.317	2.3	0.162	0.9	1.000	1.0	1.000

Cyp2d10	0.8	0.540	1.2	0.566	0.9	1.000	1.4	0.729
Cyp2d12	0.9	0.965	0.5	0.204	1.4	1.000	0.8	0.912
Cyp2d22	0.7	0.249	0.7	0.250	1.2	1.000	1.2	0.884
Cyp2d26	0.8	0.658	1.2	0.711	1.0	1.000	1.5	0.689
Cyp2d34	1.0	0.993	0.7	0.641	1.3	1.000	1.0	1.000
Cyp2d35-ps	1.6	0.742	0.9	0.934	2.2	1.000	1.2	0.995
Cyp2d36-ps	2.1	0.280	2.3	0.175	1.0	1.000	1.1	1.000
Cyp2d37-ps	1.6	0.342	1.2	0.754	1.0	1.000	0.8	0.898
Cyp2d38-ps	2.2	0.088	1.8	0.241	1.1	1.000	0.9	0.977
Cyp2d40	3.9	<0.001	5.4	<0.001	0.7	1.000	1.0	1.000
Cyp2d41-ps	1.2	0.831	0.9	0.907	1.0	1.000	0.7	0.904
Cyp2d9	0.3	0.025	0.2	0.002	1.8	1.000	1.1	1.000
Cyp2e1	0.4	0.141	0.7	0.474	1.1	1.000	1.6	0.799
Cyp2f2	0.6	0.182	0.8	0.507	0.7	1.000	0.9	0.977
Cyp2g1	2.8	0.047	3.6	0.010	0.6	1.000	0.8	0.964
Cyp2j5	0.8	0.627	0.9	0.901	1.0	1.000	1.1	0.985
Cyp2j6	0.8	0.543	1.1	0.808	0.9	1.000	1.3	0.774
Cyp2j9	0.4	0.153	0.4	0.103	0.8	1.000	0.7	0.894
Cyp2r1	1.0	0.962	1.2	0.622	0.6	0.439	0.7	0.722
Cyp2s1	0.3	0.222	4.5	0.072	0.5	1.000	7.1	0.238
Cyp2u1	0.8	0.720	0.9	0.902	0.7	1.000	0.8	0.869
Cyp39a1	0.4	0.031	0.3	0.017	1.0	1.000	0.9	0.999
Cyp3a11	0.6	0.290	0.3	0.005	0.6	0.953	0.3	0.131
Cyp3a13	1.0	1.000	1.6	0.478	0.9	1.000	1.5	0.874
Cyp3a16	20.6	0.001	128.0	<0.001	0.2	0.578	1.4	0.953
Cyp3a25	0.7	0.235	0.8	0.374	0.7	0.873	0.8	0.762
Cyp3a41a	4.8	0.047	10.8	0.002	0.6	1.000	1.4	0.941
Cyp3a41b	74.2	<0.001	181.7	<0.001	0.5	1.000	1.3	0.989
Cyp3a44	14.3	<0.001	30.5	<0.001	0.8	1.000	1.7	0.816
Cyp3a59	1.4	0.352	1.1	0.802	0.7	0.953	0.6	0.321
Cyp3a63-ps	5.1	0.047	8.5	0.007	0.9	1.000	1.5	0.926
Cyp46a1	0.6	0.547	0.4	0.117	1.0	1.000	0.6	0.816
Cyp4a10	0.7	0.494	0.8	0.721	1.2	1.000	1.4	0.782
Cyp4a12a	1.0	1.000	0.2	0.239	1.5	1.000	0.3	0.756
Cyp4a14	0.4	0.021	0.3	0.002	1.2	1.000	0.9	0.958
Cyp4a31	4.6	<0.001	5.3	<0.001	1.1	1.000	1.3	0.909
Cyp4a32	0.6	0.389	1.0	0.999	1.0	1.000	1.5	0.725
Cyp4b1	0.8	0.655	0.6	0.378	0.9	1.000	0.8	0.909
Cyp4f13	0.6	0.042	0.8	0.329	0.8	1.000	1.1	0.987
Cyp4f14	0.8	0.624	1.0	0.925	0.9	1.000	1.1	0.977
Cyp4f15	0.5	0.033	0.5	0.052	1.0	1.000	1.1	0.976
Cyp4f16	1.0	0.976	0.7	0.364	0.9	1.000	0.6	0.553
Cyp4f17	0.9	0.949	1.0	0.971	0.8	1.000	0.8	0.924
Cyp4f18	1.0	1.000	0.4	0.364	0.9	1.000	0.4	0.656
Cyp4v3	0.6	0.095	0.9	0.697	1.0	1.000	1.4	0.703
Cyp4x1	1.2	0.887	1.4	0.747	0.9	1.000	1.1	1.000
Cyp51	0.7	0.477	1.4	0.486	0.5	0.359	0.9	0.999
Cyp7a1	1.1	0.883	1.4	0.614	0.9	1.000	1.1	0.993

Cyp7b1	1.3	0.732	2.2	0.064	0.6	1.000	1.1	0.981
Cyp8b1	1.4	0.751	2.9	0.123	0.2	0.145	0.4	0.546
Ugt1a1	0.8	0.784	1.2	0.770	1.0	1.000	1.5	0.828
Ugt1a10	0.9	0.977	9.6	0.004	0.2	0.439	2.4	0.689
Ugt1a2	0.4	0.681	0.8	0.920	0.7	1.000	1.3	1.000
Ugt1a5	2.6	0.062	3.3	0.016	0.5	0.949	0.7	0.833
Ugt1a6a	1.3	0.698	1.3	0.674	0.9	1.000	0.9	0.976
Ugt1a6b	0.3	0.042	0.3	0.023	1.0	1.000	0.9	0.999
Ugt1a7c	0.8	0.893	1.8	0.449	0.8	1.000	1.8	0.799
Ugt1a8	0.3	0.496	0.2	0.230	2.9	1.000	1.6	0.974
Ugt1a9	0.9	0.970	1.8	0.237	1.1	1.000	2.1	0.440
Ugt2a2	0.7	0.836	0.9	0.938	0.9	1.000	1.1	1.000
Ugt2a3	0.8	0.364	0.9	0.869	0.9	1.000	1.1	0.943
Ugt2b1	0.8	0.435	0.8	0.360	1.2	1.000	1.1	0.924
Ugt2b34	0.8	0.709	0.9	0.884	0.9	1.000	1.1	1.000
Ugt2b35	1.3	0.522	1.2	0.648	0.9	1.000	0.9	0.954
Ugt2b36	1.2	0.815	1.5	0.447	1.1	1.000	1.4	0.833
Ugt2b37	0.7	0.800	0.8	0.876	0.5	1.000	0.5	0.797
Ugt2b38	0.2	0.139	0.0	0.013	0.5	1.000	0.1	0.408
Ugt2b5	0.9	0.841	0.9	0.799	1.1	1.000	1.0	1.000
Ugt3a1	0.7	0.510	1.0	0.973	1.0	1.000	1.4	0.809
Ugt3a2	0.9	0.957	1.4	0.579	0.9	1.000	1.3	0.915
Ugt8a	0.9	0.968	1.0	0.998	2.5	1.000	2.7	0.764
Sult1a1	0.9	0.863	1.1	0.936	1.3	1.000	1.6	0.769
Sult1b1	1.2	0.761	1.4	0.428	1.3	1.000	1.5	0.690
Sult1c2	1.0	0.981	2.1	0.067	0.9	1.000	1.9	0.467
Sult1d1	0.3	0.277	0.4	0.342	1.4	1.000	1.7	0.916
Sult1e1	0.2	0.417	0.2	0.255	9.9	0.411	13.6	0.454
Sult2a1	1.1	0.966	1.5	0.801	0.4	1.000	0.6	0.938
Sult2a2	0.6	0.750	0.5	0.557	1.0	1.000	0.8	0.989
Sult2a3	0.5	0.697	0.2	0.239	1.7	1.000	0.6	0.975
Sult2a5	0.2	0.122	0.2	0.066	1.0	1.000	0.7	0.978
Sult2a7	0.2	0.061	0.2	0.031	0.9	1.000	0.7	0.964
Sult2a8	1.1	0.934	1.5	0.548	0.9	1.000	1.2	0.982
Sult2b1	3.9	0.440	2.3	0.415	8.6	0.555	5.0	0.412
Sult3a1	3.6	0.056	12.9	<0.001	0.3	0.359	1.0	1.000
Sult3a2	53.9	<0.001	226.7	<0.001	0.2	0.605	0.9	1.000
Sult4a1	287.7	<0.001	3.5	0.333	5.2	1.000	0.1	0.129
Sult5a1	0.7	0.632	0.5	0.291	0.2	0.134	0.1	0.081
Gsta1	7.4	0.160	0.1	0.086	9.5	0.564	0.1	0.444
Gsta2	0.6	0.215	0.4	0.033	2.0	0.605	1.5	0.782
Gsta3	0.9	0.942	1.0	0.964	0.9	1.000	1.0	1.000
Gsta4	0.5	0.181	0.5	0.098	1.2	1.000	1.1	0.989
Gstcd	1.6	0.311	1.0	0.966	1.0	1.000	0.7	0.763
Gstk1	0.5	0.005	0.5	0.018	1.1	1.000	1.2	0.901
Gstm1	1.0	0.984	0.7	0.498	1.1	1.000	0.7	0.838
Gstm2	1.8	0.041	1.4	0.248	1.4	1.000	1.1	0.984
Gstm3	3.1	0.044	0.6	0.456	2.5	0.564	0.5	0.625

Gstm4	1.1	0.800	0.8	0.503	0.8	1.000	0.5	0.289
Gstm5	1.1	0.948	1.2	0.635	0.7	1.000	0.8	0.884
Gstm6	0.9	0.908	0.9	0.892	1.3	1.000	1.3	0.922
Gstm7	0.8	0.809	0.8	0.797	1.0	1.000	1.1	1.000
Gsto1	0.8	0.496	1.1	0.882	0.9	1.000	1.3	0.822
Gsto2	1.0	1.000	1.1	0.938	0.5	1.000	0.6	0.817
Gstp1	3.4	0.002	2.2	0.039	0.9	1.000	0.6	0.635
Gstp2	5.9	0.016	1.8	0.505	0.9	1.000	0.3	0.386
Gstp3	1.9	0.190	1.4	0.516	1.1	1.000	0.8	0.951
Gstt1	0.4	0.005	0.4	0.017	0.8	1.000	0.9	0.976
Gstt2	0.6	0.182	0.7	0.380	0.9	1.000	1.0	1.000
Gstt3	1.5	0.177	2.0	0.005	0.8	1.000	1.1	0.951
Abca1	1.3	0.494	1.4	0.365	1.0	1.000	1.0	1.000
Abca13	10.9	0.144	0.2	0.365	5.3	1.000	0.1	0.451
Abca17	103.3	<0.001	1779.0	<0.001	0.1	0.757	1.2	0.989
Abca2	1.2	0.873	1.4	0.579	0.8	1.000	1.0	1.000
Abca3	1.1	0.896	1.8	0.112	0.6	0.873	1.0	1.000
Abca4	2.0	0.383	0.7	0.702	1.1	1.000	0.4	0.526
Abca5	2.0	0.025	3.3	<0.001	0.8	1.000	1.3	0.768
Abca6	1.1	0.951	1.4	0.512	1.0	1.000	1.3	0.859
Abca7	0.9	0.851	0.5	0.006	1.1	1.000	0.6	0.298
Abca8a	0.7	0.493	0.5	0.158	1.4	1.000	1.0	1.000
Abca8b	1.2	0.650	1.4	0.308	0.9	1.000	1.1	0.999
Abca9	1.5	0.328	1.3	0.495	1.1	1.000	1.0	1.000
Abcb10	0.9	0.586	1.0	0.964	0.8	0.817	0.9	0.856
Abcb11	0.7	0.438	0.9	0.794	0.9	1.000	1.2	0.950
Abcb1a	0.6	0.333	0.7	0.438	0.9	1.000	1.0	1.000
Abcb1b	1.7	0.553	1.8	0.465	1.2	1.000	1.2	0.986
Abcb4	0.6	0.214	0.7	0.424	0.9	1.000	1.0	1.000
Abcb6	0.6	0.019	0.7	0.129	1.0	1.000	1.2	0.830
Abcb7	1.0	0.939	1.3	0.387	0.8	1.000	1.0	1.000
Abcb8	0.6	0.057	0.6	0.091	0.9	1.000	1.0	1.000
Abcb9	1.2	0.650	1.0	0.973	1.2	1.000	1.0	1.000
Abcc1	1.6	0.344	0.9	0.890	1.6	0.978	0.9	0.998
Abcc10	0.8	0.667	0.7	0.303	1.1	1.000	0.9	0.985
Abcc12	0.8	0.840	0.5	0.456	0.9	1.000	0.6	0.876
Abcc2	0.8	0.803	0.8	0.768	1.0	1.000	1.0	1.000
Abcc3	0.3	0.025	0.1	<0.001	1.0	1.000	0.5	0.613
Abcc4	1.4	0.698	1.0	0.977	1.5	1.000	1.2	0.978
Abcc5	1.4	0.635	0.9	0.863	1.1	1.000	0.7	0.816
Abcc6	0.6	0.364	0.7	0.395	1.1	1.000	1.2	0.955
Abcc8	0.7	0.928	1.4	0.901	1.3	1.000	2.5	0.914
Abcc9	1.0	0.979	1.2	0.667	0.9	1.000	1.2	0.941
Abcd1	1.0	0.959	1.2	0.426	0.8	1.000	1.0	1.000
Abcd2	1.1	0.947	1.4	0.650	0.7	1.000	0.9	1.000
Abcd3	0.8	0.328	0.8	0.538	0.8	0.877	0.8	0.801
Abcd4	0.8	0.406	0.6	0.078	1.2	1.000	1.0	1.000
Abce1	1.1	0.715	1.2	0.477	1.0	1.000	1.0	1.000

Abcf1	0.9	0.652	1.0	0.983	0.9	1.000	1.0	1.000
Abcf2	0.9	0.817	1.0	0.893	0.9	1.000	1.0	0.997
Abcf3	0.7	0.151	0.8	0.377	0.8	1.000	0.9	0.988
Abcg1	1.3	0.734	0.9	0.880	0.9	1.000	0.7	0.773
Abcg2	1.1	0.762	1.1	0.800	0.9	1.000	0.9	0.969
Abcg3	1.5	0.459	1.0	0.975	0.9	1.000	0.6	0.678
Abcg4	3.2	0.693	0.4	0.581	9.4	0.917	1.1	1.000
Abcg5	0.4	0.003	0.4	0.009	1.3	1.000	1.4	0.696
Abcg8	0.5	0.008	0.6	0.061	1.2	1.000	1.4	0.613
Slc10a1	0.4	0.020	0.5	0.045	0.8	1.000	1.0	1.000
Slc10a2	0.9	0.947	1.2	0.693	1.0	1.000	1.3	0.878
Slc10a3	0.9	0.681	1.0	1.000	0.8	1.000	1.0	1.000
Slc10a5	1.2	0.821	1.1	0.915	0.9	1.000	0.8	0.972
Slc10a6	0.2	0.249	0.6	0.650	1.0	1.000	2.4	0.830
Slc10a7	1.0	0.978	0.8	0.495	1.3	1.000	1.0	1.000
Slc11a1	1.5	0.567	1.2	0.836	0.7	1.000	0.6	0.689
Slc11a2	1.4	0.314	0.9	0.855	1.0	1.000	0.7	0.514
Slc12a2	0.9	0.888	1.1	0.725	1.3	0.962	1.5	0.314
Slc12a3	0.8	0.900	0.7	0.697	1.7	1.000	1.4	0.945
Slc12a4	0.9	0.914	0.8	0.408	1.0	1.000	0.8	0.820
Slc12a5	0.7	0.792	0.5	0.446	0.9	1.000	0.7	0.916
Slc12a6	1.1	0.789	0.9	0.662	1.1	1.000	0.9	0.901
Slc12a7	0.6	0.153	0.6	0.066	0.8	1.000	0.8	0.768
Slc12a8	1.2	0.909	1.0	1.000	1.3	1.000	1.1	1.000
Slc12a9	0.7	0.472	0.9	0.883	1.1	1.000	1.4	0.768
Slc13a2	1.4	0.779	2.6	0.158	0.5	1.000	1.0	1.000
Slc13a3	0.4	0.068	0.5	0.244	0.7	1.000	0.9	0.997
Slc13a4	0.3	0.250	0.6	0.672	0.6	1.000	1.2	1.000
Slc13a5	0.4	0.216	0.2	0.070	0.8	1.000	0.5	0.788
Slc14a1	1.4	0.878	6.5	0.066	0.5	1.000	2.5	0.768
Slc14a2	1.4	0.947	3.9	0.479	0.2	1.000	0.5	0.941
Slc15a1	1.2	0.915	0.9	0.938	1.2	1.000	0.8	0.995
Slc15a2	1.5	0.736	0.3	0.229	2.3	1.000	0.5	0.809
Slc15a3	1.8	0.316	1.0	0.994	0.9	1.000	0.5	0.550
Slc15a4	0.9	0.761	0.6	0.072	1.2	1.000	0.8	0.874
Slc15a5	0.5	0.404	0.7	0.786	0.5	1.000	0.8	0.986
Slc16a1	2.0	0.018	1.6	0.142	0.9	1.000	0.7	0.625
Slc16a10	0.5	0.051	0.7	0.283	1.1	1.000	1.5	0.635
Slc16a11	0.9	0.897	1.0	0.995	0.6	0.953	0.7	0.768
Slc16a12	0.5	0.088	0.7	0.401	0.9	1.000	1.2	0.945
Slc16a13	2.2	0.136	1.6	0.408	0.5	0.940	0.4	0.347
Slc16a2	0.7	0.628	1.0	0.974	0.8	1.000	1.1	0.999
Slc16a3	3.3	0.107	1.1	0.959	2.2	1.000	0.7	0.945
Slc16a4	0.9	0.900	0.6	0.341	1.2	1.000	0.8	0.946
Slc16a5	1.0	0.978	1.2	0.781	1.1	1.000	1.2	0.917
Slc16a6	16.2	<0.001	14.4	<0.001	1.1	1.000	1.0	1.000
Slc16a7	0.7	0.458	0.8	0.633	1.1	1.000	1.2	0.854
Slc16a9	1.1	0.908	1.2	0.752	0.9	1.000	1.0	1.000

Slc17a1	0.7	0.687	0.6	0.507	1.0	1.000	0.9	0.993
Slc17a2	0.3	0.057	0.2	0.009	0.9	1.000	0.6	0.816
Slc17a3	0.7	0.336	0.9	0.685	0.9	1.000	1.1	0.987
Slc17a4	1.7	0.415	1.5	0.493	1.3	1.000	1.1	0.996
Slc17a5	0.9	0.930	1.1	0.872	1.0	1.000	1.2	0.930
Slc17a8	1.9	0.375	0.6	0.519	1.0	1.000	0.4	0.383
Slc17a9	1.4	0.593	1.2	0.757	0.9	1.000	0.8	0.950
Slc18a1	1.4	0.575	1.1	0.845	1.3	1.000	1.1	1.000
Slc18a2	1.0	0.977	1.0	0.984	1.0	1.000	0.9	1.000
Slc18b1	0.9	0.915	1.1	0.939	0.8	1.000	1.0	1.000
Slc19a1	0.6	0.178	0.8	0.557	1.1	1.000	1.4	0.761
Slc19a2	0.7	0.156	0.7	0.198	0.9	1.000	0.9	0.996
Slc19a3	0.4	0.650	3.7	0.483	0.4	1.000	3.7	0.816
Slc1a1	1.2	0.900	1.4	0.800	0.9	1.000	1.1	1.000
Slc1a2	1.5	0.699	2.1	0.313	1.4	1.000	2.0	0.756
Slc1a3	1.5	0.825	0.6	0.660	1.4	1.000	0.5	0.895
Slc1a4	1.8	0.140	1.0	1.000	1.1	1.000	0.6	0.533
Slc1a5	0.7	0.616	0.5	0.141	1.3	1.000	0.9	0.987
Slc1a6	0.5	0.714	0.3	0.475	0.9	1.000	0.5	0.951
Slc20a1	0.6	0.059	0.7	0.141	1.1	1.000	1.2	0.832
Slc20a2	1.4	0.188	1.5	0.113	1.0	1.000	1.0	1.000
Slc22a1	0.8	0.595	0.9	0.847	1.0	1.000	1.2	0.953
Slc22a13b-ps	0.4	0.487	0.5	0.528	0.9	1.000	1.0	1.000
Slc22a15	0.5	0.008	0.5	0.028	1.1	1.000	1.2	0.869
Slc22a17	1.0	0.990	0.9	0.906	0.9	1.000	0.8	0.975
Slc22a18	0.7	0.294	0.9	0.810	1.0	1.000	1.4	0.768
Slc22a2	260.5	<0.001	561.5	<0.001	0.4	1.000	0.8	0.984
Slc22a21	0.3	0.083	1.9	0.337	0.5	0.889	2.8	0.433
Slc22a23	1.4	0.270	1.6	0.080	1.0	1.000	1.1	0.967
Slc22a26	0.6	0.352	1.0	1.000	0.7	1.000	1.1	0.985
Slc22a27	0.5	0.126	0.5	0.101	0.6	0.992	0.6	0.665
Slc22a28	2.9	0.100	2.8	0.102	1.3	1.000	1.2	0.976
Slc22a29	0.2	0.075	0.2	0.072	0.9	1.000	0.9	1.000
Slc22a3	0.9	0.930	0.9	0.890	1.6	1.000	1.6	0.767
Slc22a30	1.1	0.852	1.3	0.317	0.9	1.000	1.1	0.996
Slc22a4	0.5	0.194	0.8	0.825	0.8	1.000	1.3	0.917
Slc22a5	0.8	0.566	0.9	0.709	1.0	1.000	1.1	0.951
Slc22a6	0.4	0.654	0.2	0.449	1.7	1.000	1.0	1.000
Slc22a7	1.3	0.713	3.1	0.004	0.5	0.437	1.1	0.975
Slc22a8	0.3	0.567	0.0	0.108	1.9	1.000	0.3	0.890
Slc23a1	1.0	1.000	1.2	0.682	0.7	1.000	0.9	0.969
Slc23a2	0.6	0.194	0.7	0.336	0.9	1.000	1.0	1.000
Slc23a3	1.1	0.975	0.6	0.764	2.7	1.000	1.5	0.974
Slc23a4	1.6	0.654	0.4	0.269	1.7	1.000	0.4	0.681
Slc24a3	9.5	<0.001	6.6	<0.001	1.3	1.000	0.9	0.969
Slc24a5	0.7	0.690	0.9	0.920	0.6	1.000	0.8	0.962
Slc25a1	0.7	0.153	0.8	0.343	0.7	0.811	0.8	0.786
Slc25a10	0.7	0.080	0.8	0.336	0.9	1.000	1.0	1.000

Slc25a11	0.7	0.016	0.7	0.029	1.0	1.000	1.0	1.000
Slc25a12	1.0	0.989	0.9	0.845	0.9	1.000	0.8	0.901
Slc25a13	0.7	0.220	0.8	0.529	1.0	1.000	1.2	0.880
Slc25a14	1.1	0.944	0.7	0.420	1.1	1.000	0.7	0.768
Slc25a15	0.8	0.688	0.9	0.833	1.3	1.000	1.4	0.713
Slc25a16	1.0	0.995	1.1	0.796	1.1	1.000	1.2	0.857
Slc25a17	1.0	0.943	0.9	0.500	0.9	1.000	0.8	0.654
Slc25a18	1.4	0.820	0.4	0.257	2.5	0.940	0.7	0.945
Slc25a19	0.8	0.291	0.6	0.015	1.1	1.000	0.9	0.857
Slc25a20	1.0	0.975	1.0	0.908	1.0	1.000	0.9	0.946
Slc25a21	0.7	0.286	0.6	0.182	1.0	1.000	0.9	0.978
Slc25a22	1.0	0.990	0.7	0.221	1.4	0.757	1.0	0.999
Slc25a23	0.7	0.245	1.0	1.000	0.8	1.000	1.2	0.924
Slc25a24	1.1	0.862	1.2	0.703	1.1	1.000	1.2	0.945
Slc25a25	1.1	0.885	1.1	0.861	1.6	1.000	1.6	0.701
Slc25a26	1.1	0.849	1.1	0.732	0.9	1.000	1.0	1.000
Slc25a27	0.8	0.836	0.4	0.229	1.8	1.000	1.0	1.000
Slc25a28	0.6	0.007	0.7	0.024	0.9	1.000	0.9	0.953
Slc25a29	0.8	0.585	1.0	0.977	1.0	1.000	1.3	0.816
Slc25a3	0.8	0.504	0.8	0.231	1.0	1.000	0.9	0.947
Slc25a30	1.7	0.128	2.2	0.010	0.5	0.315	0.7	0.639
Slc25a31	0.4	0.521	0.4	0.589	1.0	1.000	1.2	1.000
Slc25a32	0.8	0.457	0.8	0.381	1.3	1.000	1.3	0.722
Slc25a33	1.4	0.250	1.5	0.147	0.9	1.000	0.9	0.999
Slc25a34	1.0	1.000	1.1	0.936	1.0	1.000	1.1	0.998
Slc25a35	0.7	0.608	0.9	0.890	0.9	1.000	1.2	0.985
Slc25a36	1.2	0.683	1.5	0.299	1.1	1.000	1.3	0.837
Slc25a37	0.5	0.012	0.6	0.027	0.9	1.000	1.0	1.000
Slc25a38	0.9	0.631	0.9	0.609	1.1	1.000	1.1	0.869
Slc25a39	0.7	0.070	0.8	0.298	0.9	1.000	1.0	0.994
Slc25a4	1.0	0.948	0.8	0.628	1.0	1.000	0.8	0.799
Slc25a40	1.1	0.943	0.9	0.833	0.9	1.000	0.8	0.854
Slc25a42	0.8	0.396	0.7	0.320	0.9	1.000	0.9	0.912
Slc25a43	0.8	0.876	0.5	0.329	0.8	1.000	0.4	0.625
Slc25a44	1.1	0.895	1.1	0.701	1.0	1.000	1.0	1.000
Slc25a45	0.9	0.916	1.0	0.983	0.8	1.000	0.9	0.913
Slc25a46	0.9	0.787	1.1	0.728	1.0	1.000	1.2	0.800
Slc25a47	0.9	0.746	0.9	0.861	1.2	1.000	1.3	0.817
Slc25a48	0.6	0.189	0.7	0.387	1.2	1.000	1.4	0.753
Slc25a5	0.8	0.388	0.8	0.271	0.9	1.000	0.9	0.886
Slc25a51	0.6	0.026	0.3	<0.001	1.8	0.181	1.1	0.951
Slc25a53	1.2	0.893	0.7	0.703	1.4	1.000	0.8	0.979
Slc26a1	0.8	0.793	1.1	0.903	0.9	1.000	1.1	0.988
Slc26a10	0.4	0.079	0.3	0.006	1.3	1.000	0.9	0.988
Slc26a11	0.9	0.864	0.7	0.206	1.1	1.000	0.8	0.833
Slc26a2	1.2	0.580	1.0	0.994	1.1	1.000	0.9	0.961
Slc26a3	1.4	0.899	7.5	0.104	0.9	1.000	4.8	0.601
Slc26a4	0.1	0.009	0.2	0.016	1.2	1.000	1.6	0.947

Slc26a6	1.1	0.955	1.9	0.062	0.5	0.536	1.0	1.000
Slc26a7	0.1	0.150	3.3	0.516	0.1	0.615	3.3	0.838
Slc26a8	0.4	0.450	0.7	0.800	0.9	1.000	1.6	0.950
Slc27a1	0.7	0.210	0.6	0.018	1.3	1.000	1.0	1.000
Slc27a2	0.7	0.547	0.7	0.530	1.0	1.000	1.0	1.000
Slc27a3	1.9	0.334	0.8	0.754	2.7	0.438	1.1	0.998
Slc27a4	1.0	0.934	1.2	0.517	0.9	1.000	1.1	0.945
Slc27a5	0.6	0.242	0.9	0.837	0.8	1.000	1.2	0.943
Slc27a6	1.8	0.632	0.8	0.920	1.5	1.000	0.7	0.951
Slc28a1	0.4	0.347	0.6	0.572	0.8	1.000	1.2	1.000
Slc28a2	1.3	0.804	1.3	0.788	1.0	1.000	1.0	1.000
Slc28a3	0.4	0.702	0.7	0.837	4.2	0.875	7.3	0.597
Slc29a1	0.7	0.137	0.7	0.166	1.1	1.000	1.2	0.888
Slc29a2	0.6	0.285	0.4	0.075	1.0	1.000	0.7	0.888
Slc29a3	1.2	0.806	1.5	0.321	0.8	1.000	1.1	0.993
Slc29a4	0.9	0.996	0.2	0.287	2.8	1.000	0.7	0.983
Slc2a1	1.0	0.990	0.9	0.940	0.9	1.000	0.8	0.946
Slc2a10	0.9	0.977	1.4	0.800	0.8	1.000	1.2	0.999
Slc2a12	0.7	0.737	3.2	0.106	0.4	0.761	1.6	0.847
Slc2a13	1.3	0.817	0.6	0.495	1.9	0.995	1.0	1.000
Slc2a2	0.5	0.233	0.6	0.438	1.1	1.000	1.4	0.911
Slc2a3	1.9	0.495	3.4	0.116	2.8	0.873	5.0	0.242
Slc2a4	0.3	0.257	0.4	0.296	2.0	1.000	2.3	0.739
Slc2a4rg-ps	0.6	0.453	0.4	0.071	1.2	1.000	0.8	0.914
Slc2a5	0.9	0.948	1.2	0.796	0.9	1.000	1.1	0.991
Slc2a6	4.4	0.144	0.6	0.668	1.7	1.000	0.2	0.478
Slc2a7	1.0	1.000	1.0	1.000	1.0	1.000	1.0	1.000
Slc2a8	1.0	0.919	1.0	0.970	0.9	1.000	1.0	0.998
Slc2a9	0.7	0.404	0.8	0.550	0.9	1.000	1.0	1.000
Slc30a1	1.3	0.505	1.6	0.051	1.0	1.000	1.3	0.799
Slc30a10	1.7	0.622	1.3	0.854	1.1	1.000	0.8	0.984
Slc30a2	2.0	0.661	2.1	0.775	0.3	1.000	0.3	0.747
Slc30a3	0.4	0.499	0.4	0.533	0.5	1.000	0.6	0.946
Slc30a4	1.4	0.351	1.4	0.333	1.1	1.000	1.1	0.985
Slc30a5	1.1	0.870	1.0	0.975	1.0	1.000	1.0	1.000
Slc30a6	1.2	0.552	1.2	0.454	1.0	1.000	1.0	1.000
Slc30a7	1.0	0.973	0.9	0.921	0.8	1.000	0.8	0.901
Slc30a9	0.8	0.245	0.8	0.181	1.0	1.000	1.0	1.000
Slc31a1	0.9	0.851	1.1	0.739	0.9	1.000	1.1	0.960
Slc31a2	1.1	0.797	1.1	0.751	0.9	1.000	0.9	0.971
Slc33a1	1.4	0.280	1.5	0.076	1.0	1.000	1.1	0.943
Slc34a2	2.6	0.176	2.0	0.403	0.6	1.000	0.5	0.694
Slc34a3	1.0	1.000	1.0	1.000	1.0	1.000	1.0	1.000
Slc35a1	0.9	0.827	1.1	0.578	0.8	0.999	1.0	1.000
Slc35a2	1.0	0.930	1.0	0.947	1.0	1.000	1.1	0.988
Slc35a3	1.0	0.937	1.1	0.709	1.0	1.000	1.1	0.978
Slc35a4	0.9	0.938	1.3	0.506	1.0	1.000	1.5	0.689
Slc35a5	1.1	0.800	1.0	1.000	1.1	1.000	1.0	1.000

Slc35b1	1.8	0.006	2.5	<0.001	1.0	1.000	1.3	0.617
Slc35b2	1.0	0.977	1.5	0.089	0.9	1.000	1.3	0.629
Slc35b3	1.1	0.771	1.3	0.272	0.8	1.000	1.0	0.996
Slc35b4	1.0	0.984	1.4	0.288	0.9	1.000	1.4	0.753
Slc35c1	1.3	0.254	1.6	0.029	0.9	1.000	1.1	0.953
Slc35c2	1.7	0.003	2.0	<0.001	0.9	1.000	1.0	1.000
Slc35d1	1.2	0.546	1.1	0.735	0.8	1.000	0.8	0.595
Slc35d2	1.2	0.542	1.5	0.131	0.8	1.000	1.0	1.000
Slc35d3	3.5	0.641	5.8	0.488	0.4	1.000	0.6	0.984
Slc35e1	1.1	0.628	1.0	0.919	1.1	1.000	1.0	0.997
Slc35e2	1.0	0.944	1.0	0.964	1.3	0.817	1.3	0.702
Slc35e3	0.8	0.483	0.7	0.158	0.9	1.000	0.8	0.745
Slc35e4	1.8	0.451	1.3	0.794	1.2	1.000	0.9	0.988
Slc35f1	1.8	0.798	0.8	0.937	1.7	1.000	0.7	0.994
Slc35f2	1.1	0.979	1.0	0.995	0.9	1.000	0.8	0.999
Slc35f3	1.0	1.000	2.3	0.532	0.8	1.000	1.8	0.917
Slc35f5	1.3	0.458	1.1	0.781	1.0	1.000	0.9	0.951
Slc35f6	1.0	0.942	1.1	0.876	1.0	1.000	1.1	0.971
Slc35g1	0.9	0.742	1.0	0.946	1.1	1.000	1.2	0.876
Slc35g2	0.7	0.754	0.7	0.684	1.6	1.000	1.5	0.865
Slc36a1	2.3	0.001	2.7	<0.001	0.9	1.000	1.0	1.000
Slc36a2	0.1	0.266	0.8	0.915	0.9	1.000	4.6	0.756
Slc36a4	1.1	0.901	1.2	0.598	0.9	1.000	1.0	1.000
Slc37a1	11.6	<0.001	6.2	<0.001	1.5	1.000	0.8	0.946
Slc37a2	1.2	0.832	0.7	0.424	0.8	1.000	0.5	0.350
Slc37a3	1.4	0.227	1.4	0.208	1.0	1.000	1.0	1.000
Slc37a4	0.5	0.094	0.6	0.135	1.3	1.000	1.4	0.809
Slc38a1	1.0	0.988	1.3	0.605	1.1	1.000	1.3	0.835
Slc38a10	1.3	0.351	1.6	0.062	0.8	1.000	1.0	1.000
Slc38a11	0.5	0.595	0.7	0.791	0.9	1.000	1.3	0.991
Slc38a2	1.1	0.704	1.3	0.202	1.4	0.322	1.6	0.107
Slc38a3	0.8	0.551	0.9	0.715	1.0	1.000	1.1	0.971
Slc38a4	0.3	<0.001	0.3	<0.001	1.1	1.000	1.2	0.951
Slc38a5	1.2	0.955	0.4	0.465	2.8	1.000	1.0	1.000
Slc38a6	0.9	0.914	0.9	0.751	1.1	1.000	1.0	1.000
Slc38a7	0.7	0.149	0.9	0.609	0.9	1.000	1.1	0.945
Slc38a8	1.2	0.967	0.9	0.958	3.3	1.000	2.5	0.865
Slc38a9	1.0	0.963	0.9	0.742	1.1	1.000	0.9	0.973
Slc39a1	1.0	0.998	1.2	0.507	0.9	1.000	1.1	0.965
Slc39a10	1.3	0.439	0.7	0.344	1.4	1.000	0.7	0.722
Slc39a11	1.4	0.180	2.0	<0.001	0.9	1.000	1.3	0.626
Slc39a13	1.1	0.728	1.1	0.853	1.0	1.000	1.0	1.000
Slc39a14	2.3	0.013	2.1	0.022	1.5	0.978	1.4	0.775
Slc39a2	0.6	0.522	0.5	0.208	1.1	1.000	0.9	0.988
Slc39a3	1.1	0.749	1.0	0.877	0.9	1.000	0.9	0.794
Slc39a4	0.9	0.817	0.7	0.358	0.8	1.000	0.6	0.603
Slc39a5	1.5	0.680	1.2	0.878	1.1	1.000	0.9	0.998
Slc39a6	1.0	0.990	1.0	1.000	1.3	1.000	1.3	0.843

Slc39a7	1.1	0.762	1.2	0.461	0.9	1.000	0.9	0.975
Slc39a8	1.0	0.996	1.1	0.817	1.0	1.000	1.2	0.949
Slc39a9	1.1	0.707	1.2	0.579	1.0	1.000	1.1	0.978
Slc3a1	1.0	1.000	1.8	0.469	0.7	1.000	1.2	0.985
Slc3a2	0.8	0.448	0.9	0.719	1.0	1.000	1.1	0.936
Slc40a1	1.5	0.292	1.6	0.180	0.7	1.000	0.8	0.837
Slc41a1	1.0	0.997	1.2	0.671	0.7	1.000	0.9	0.943
Slc41a2	30.2	<0.001	28.1	<0.001	1.1	1.000	1.0	1.000
Slc41a3	4.3	0.001	3.5	0.002	1.9	0.781	1.5	0.753
Slc43a1	2.6	0.037	3.1	0.010	1.1	1.000	1.3	0.934
Slc43a2	1.1	0.895	1.0	0.982	0.9	1.000	0.9	0.967
Slc43a3	0.8	0.493	0.8	0.334	1.0	1.000	0.9	0.962
Slc44a1	1.1	0.751	1.2	0.425	0.9	1.000	1.0	1.000
Slc44a2	0.9	0.721	0.8	0.477	1.0	1.000	0.9	0.943
Slc44a3	0.9	0.908	0.6	0.416	0.9	1.000	0.7	0.803
Slc44a4	0.6	0.844	2.6	0.389	1.6	1.000	6.9	0.404
Slc45a3	3.0	0.001	2.1	0.027	1.2	1.000	0.9	0.948
Slc45a4	1.2	0.706	1.4	0.343	0.7	1.000	0.8	0.912
Slc46a1	0.9	0.608	1.1	0.570	0.7	0.593	1.0	0.995
Slc46a3	0.5	0.090	0.9	0.866	0.7	1.000	1.4	0.844
Slc47a1	0.9	0.922	1.0	0.975	0.9	1.000	1.0	1.000
Slc48a1	1.1	0.749	1.0	1.000	1.0	1.000	0.9	0.914
Slc4a1	8.0	0.002	24.0	<0.001	0.9	1.000	2.6	0.415
Slc4a11	0.6	0.755	0.6	0.742	0.6	1.000	0.6	0.944
Slc4a1ap	0.8	0.268	0.7	0.074	1.0	1.000	0.9	0.869
Slc4a2	0.9	0.818	0.8	0.433	1.0	1.000	0.9	0.938
Slc4a3	0.6	0.469	0.9	0.856	0.9	1.000	1.3	0.930
Slc4a4	0.7	0.439	0.8	0.517	1.1	1.000	1.1	0.967
Slc4a5	1.3	0.926	0.3	0.436	2.9	1.000	0.6	0.984
Slc4a7	1.1	0.862	1.1	0.699	1.0	1.000	1.1	0.982
Slc4a8	1.1	0.978	0.3	0.272	0.9	1.000	0.3	0.557
Slc4a9	0.1	0.025	0.1	0.001	3.9	0.359	1.9	0.914
Slc50a1	0.6	0.144	0.8	0.703	0.9	1.000	1.3	0.816
Slc51a	0.3	0.381	0.3	0.424	0.4	1.000	0.4	0.795
Slc51b	1.4	0.835	0.9	0.943	0.8	1.000	0.5	0.820
Slc52a2	0.6	0.178	0.6	0.112	0.8	1.000	0.7	0.762
Slc52a3	0.9	0.931	1.6	0.605	1.1	1.000	2.0	0.762
Slc5a1	0.6	0.551	0.8	0.856	1.1	1.000	1.6	0.897
Slc5a11	0.5	0.758	0.5	0.734	0.4	1.000	0.3	0.868
Slc5a3	1.2	0.779	0.9	0.861	1.1	1.000	0.8	0.949
Slc5a4b	0.7	0.821	0.9	0.966	1.2	1.000	1.7	0.938
Slc5a5	1.8	0.786	2.1	0.703	0.6	1.000	0.7	0.999
Slc5a6	0.5	0.130	0.6	0.166	1.2	1.000	1.3	0.912
Slc5a9	8.5	0.247	0.4	0.685	8.4	0.908	0.4	0.912
Slc6a1	2.1	0.629	0.9	0.971	0.7	1.000	0.3	0.739
Slc6a12	1.1	0.952	1.6	0.137	0.9	1.000	1.4	0.718
Slc6a13	0.8	0.699	0.9	0.818	0.9	1.000	1.0	1.000
Slc6a14	0.5	0.769	3.5	0.348	0.8	1.000	5.4	0.575

Slc6a15	0.5	0.809	1.1	0.997	0.5	1.000	1.2	1.000
Slc6a16	0.3	0.253	0.4	0.249	1.4	1.000	1.5	0.941
Slc6a17	0.9	0.993	0.6	0.731	2.4	1.000	1.5	0.978
Slc6a18	2.9	0.637	7.4	0.293	1.0	1.000	2.6	0.901
Slc6a19	0.1	0.369	0.2	0.320	1.6	1.000	2.0	0.975
Slc6a2	4.6	0.452	0.9	0.953	6.0	0.979	1.1	1.000
Slc6a20b	1.6	0.646	0.6	0.525	1.5	1.000	0.6	0.799
Slc6a4	1.8	0.513	1.4	0.783	0.6	1.000	0.5	0.744
Slc6a6	1.2	0.713	1.6	0.159	0.8	1.000	1.0	1.000
Slc6a8	0.9	0.836	1.3	0.616	0.9	1.000	1.4	0.856
Slc6a9	6.1	<0.001	9.0	<0.001	0.7	1.000	1.1	1.000
Slc7a1	1.4	0.606	1.1	0.915	1.6	1.000	1.2	0.956
Slc7a10	0.3	0.619	0.4	0.559	2.8	1.000	4.4	0.763
Slc7a11	2.0	0.478	1.2	0.861	2.2	1.000	1.4	0.950
Slc7a14	0.6	0.680	0.3	0.273	1.5	1.000	0.7	0.978
Slc7a15	5.0	0.102	19.1	0.010	0.1	0.554	0.4	0.786
Slc7a2	0.4	0.013	0.5	0.058	1.3	1.000	1.6	0.625
Slc7a3	1.8	0.779	0.6	0.776	2.5	1.000	0.8	1.000
Slc7a4	1.1	0.933	1.0	1.000	0.9	1.000	0.8	0.971
Slc7a5	0.8	0.833	0.9	0.848	1.1	1.000	1.2	0.969
Slc7a6	0.5	0.451	2.3	0.214	1.0	1.000	4.6	0.262
Slc7a6os	0.9	0.658	0.8	0.243	1.0	1.000	0.9	0.947
Slc7a7	5.0	<0.001	2.9	0.003	1.1	1.000	0.6	0.601
Slc7a8	1.6	0.380	1.3	0.649	0.9	1.000	0.7	0.841
Slc7a9	8.0	0.255	14.3	0.138	0.6	1.000	1.1	1.000
Slc8a1	3.1	0.236	2.1	0.486	0.7	1.000	0.5	0.803
Slc8b1	0.5	0.023	0.6	0.055	0.8	1.000	0.9	0.958
Slc9a1	0.9	0.792	0.8	0.421	1.1	1.000	1.0	1.000
Slc9a2	0.8	0.965	13.0	0.056	0.3	1.000	4.9	0.575
Slc9a3	0.4	0.766	74.3	0.005	0.1	1.000	23.5	0.238
Slc9a3r1	0.6	0.055	0.7	0.229	0.9	1.000	1.1	0.951
Slc9a3r2	0.6	0.067	0.6	0.102	0.9	1.000	1.0	1.000
Slc9a5	1.0	0.998	0.7	0.518	1.2	1.000	0.8	0.951
Slc9a6	1.1	0.872	1.0	1.000	1.0	1.000	0.9	0.997
Slc9a7	1.7	0.429	2.3	0.141	0.6	1.000	0.9	0.979
Slc9a8	0.7	0.197	0.8	0.267	0.9	1.000	0.9	0.943
Slc9a9	1.3	0.567	1.1	0.861	0.9	1.000	0.8	0.855
Slc9b1	3.2	0.335	5.5	0.152	0.5	1.000	0.8	1.000
Slc9b2	1.3	0.734	1.2	0.795	1.1	1.000	1.0	1.000
Slco1a1	1.6	0.585	2.0	0.319	0.5	1.000	0.6	0.856
Slco1a4	0.4	0.045	0.4	0.063	1.1	1.000	1.2	0.945
Slco1a6	3.9	0.339	5.1	0.192	0.9	1.000	1.2	1.000
Slco1b2	0.5	0.316	0.6	0.375	1.1	1.000	1.2	0.964
Slco2a1	1.1	0.883	1.3	0.562	0.9	1.000	1.0	1.000
Slco2b1	0.9	0.806	1.1	0.859	1.0	1.000	1.2	0.869
Slco3a1	1.0	0.983	0.8	0.635	1.0	1.000	0.8	0.894
Slco4a1	0.5	0.475	0.6	0.524	1.1	1.000	1.3	0.976
Slco4c1	4.8	0.519	17.8	0.035	2.6	1.000	9.6	0.376

Slco5a1	1.8	0.479	0.3	0.180	2.1	0.982	0.4	0.636

Supplemental Table S3. Plasma concentrations of bile acids in CVNP, CVP, GFNP, and GFP female C57BL/6 mice. Data shown are means ± SD of 5-6 mice.

Bile Acids	Plasma Concentration (ng/ml)			
	CVNP	CVP	GFNP	GFP
Primary Bile Acids				
α-MCA	108.2 ± 78.4	714.2 ± 880.9	13 ± 17.7	7.7 ± 7.8
T-α-MCA	25.6 ± 4.4	465.7 ± 850.9	3550.7 ± 6505.9	998.5 ± 1451.9
β-MCA	340.5 ± 379.4	1112.7 ± 844.1	140.8 ± 175.5	65.7 ± 53.3
T-β-MCA	36.7 ± 17.7	2674.4 ± 5832.7	22884.7 ± 31772.6	9222.2 ± 10750
CA	393 ± 269.8	5678.6 ± 7628.4	49.6 ± 22.2	37.3 ± 4
CDCA	148.4 ± 36.3	892.2 ± 921.5	128.8 ± 52.1	100 ± 4.7
TCA	173.5 ± 85.8	2483.7 ± 4355.4	9636.8 ± 15368.9	3312 ± 5091.4
TCDCA	15.2 ± 5.5	60.5 ± 85.4	1081.5 ± 2185.7	244.4 ± 378.9
UDCA	68.2 ± 52	688.7 ± 762	3.3 ± 6	1.8 ± 2.3
TUDCA	14.7 ± 4.4	81.5 ± 149.2	797.1 ± 1651.7	268.3 ± 487
Secondary Bile Acids				
ω-MCA	351.5 ± 293.7	2101 ± 1967	0.1 ± 0	0.1 ± 0
T-ω-MCA	334.6 ± 104.4	2897.7 ± 5149.8	0.1 ± 0	0.1 ± 0
DCA	70.9 ± 69.7	478.1 ± 440.4	3.6 ± 7.3	6.3 ± 13.8
TDCA	46.2 ± 31.5	38 ± 16.6	1.7 ± 0	1.7 ± 0
MDCA	47.5 ± 4.2	80 ± 41	43.7 ± 0.6	43.5 ± 0.6
HDCA	225.8 ± 54.9	2037.8 ± 1492.2	29 ± 24.1	8 ± 6.1
THDCA	27.2 ± 10.2	86 ± 89.8	64.9 ± 67.2	31.1 ± 29.3
LCA	81.9 ± 33.8	160 ± 119.4	10.4 ± 9.1	1.9 ± 4
TLCA	19 ± 21.6	94.1 ± 117.3	8.3 ± 20.1	0.1 ± 0

Supplemental Table S4. Plasma concentrations of steroid hormones in CVNP, CVP, GFNP, and GFP female C57BL/6 mice. Data shown are means ± SD of 5-6 mice.

Steroid Hormones	Plasma Concentration (ng/ml)			
	CVNP	CVP	GFNP	GFP
11-deoxycorticosterone	43.5 ± 10.5	197.7 ± 100	107.8 ± 45.4	396.2 ± 112.7
17-OH-pregnenolone	621 ± 211	3329.6 ± 1731.1	910.1 ± 247.4	6186.2 ± 1770.4
17-OH-progesterone	43.4 ± 11.5	198.4 ± 106.9	112.5 ± 37.8	419.9 ± 129.4
Aldosterone	0.1 ± 0	1.7 ± 2.7	0.7 ± 1.5	10.3 ± 3.5
Corticosterone	693.4 ± 214.1	4089 ± 2135.5	1201 ± 252.4	7562.3 ± 2327.3
Cortisol	46.1 ± 19.7	1247.6 ± 741.7	80.8 ± 34.3	1749 ± 427.5
Cortisone	0.1 ± 0	6 ± 5.8	0.1 ± 0	7.4 ± 3.5
DHEA	2.3 ± 2.8	1.3 ± 1.3	0.3 ± 0.3	0.1 ± 0
Estradiol	67.8 ± 37.1	30.9 ± 10.1	65.3 ± 66.2	124.5 ± 128
Estrone	23 ± 19.2	8.6 ± 10.4	28.9 ± 53	20.4 ± 20.8
Pregnenolone	0.1 ± 0	2.5 ± 4.5	1.4 ± 2.4	6.7 ± 4.8
Progesterone	18.6 ± 15.7	577.2 ± 235.8	28.7 ± 47.3	458.5 ± 192.3
Testosterone	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0