CYP2D6 activity is correlated with changes in plasma concentrations of taurocholic acid during pregnancy and postpartum in CYP2D6 extensive metabolizers

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Running Title: CYP2D6 activity is correlated with bile acid concentrations.

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Abbreviations: all-trans-retinoic acid (atRA), bile acid-CoA:amino acid N-acyltransferase (BAAT), cholic acid (CA), chenodeoxycholic acid (CDCA), Cytochrome P450 (CYP) 2D6 (CYP2D6), deoxycholic acid (DCA), dextromethorphan (DM), dextrophan (DX), farnesoid X receptor (FXR), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), and glycodeoxycholic acid (GDCA), hepatocyte nuclear factor 4 alpha (HNF4α), interquartile range (IQR), liver receptor homologue-1 (LRH-1), small heterodimer partner (SHP), Study Day 1 (SD1), Study Day 2 (SD2), Study Day 3 (SD3) taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCNA), taurodeoxycholic acid (TDCA), ultra-high-performance liquid chromatography mass spectrometry (UHPLC-MS/MS)
Abstract

Cytochrome P450 2D6 (CYP2D6) is involved in the metabolism of > 20% of marketed drugs. CYP2D6 expression and activity exhibit high interindividual variability and is induced during pregnancy. The farnesoid X receptor (FXR) is a transcriptional regulator of CYP2D6 that is activated by bile acids. In pregnancy, elevated plasma bile acid concentrations are associated with maternal and fetal risks. However, modest changes in bile acid concentrations may occur during healthy pregnancy thereby altering FXR signaling. A previous study demonstrated that hepatic tissue concentrations of bile acids positively correlated with the hepatic mRNA expression of CYP2D6. This study sought to characterize the plasma bile acid metabolome in healthy women (n=47) during mid-pregnancy (25-28 weeks gestation) and ≥ 3 months postpartum, and to determine if plasma bile acids correlate with CYP2D6 activity. It is hypothesized that during pregnancy plasma bile acids would favor less hydrophobic bile acids (cholic acid vs. chenodeoxycholic acid), and that plasma concentrations of cholic acid and its conjugates would positively correlate with the urinary ratio of dextrorphan / dextromethorphan. At 25-28 weeks gestation, taurine conjugated bile acids comprised 23% of the quantified serum bile acids compared to 7% ≥ 3 months postpartum. Taurocholic acid positively associated with the urinary ratio of dextrorphan / dextromethorphan, a biomarker of CYP2D6 activity. Collectively, these results confirm that the bile acid plasma metabolome differs between pregnancy and postpartum and provide evidence that taurocholic acid may impact CYP2D6 activity during pregnancy.
Significance Statement (43/80 words)

Bile acid homeostasis is altered in pregnancy and plasma concentrations of taurocholic acid positively correlate with CYP2D6 activity. Differences between plasma and/or tissue concentrations of FXR ligands, such as bile acids, may contribute to the high interindividual variability in CYP2D6 expression and activity.
Introduction

Cytochrome P450 2D6 (CYP2D6) is a drug metabolizing enzyme responsible for the metabolism of >20% of marketed drugs in the United States (Pan et al., 2017). CYP2D6 expression and activity exhibit high interindividual variability that is attributed to genetic determinants such as allelic copy number and polymorphisms, regulation by transcription factors, and post-transcriptional regulatory mechanisms (Pan et al., 2017; Gaedigk et al., 2018). The mechanisms that determine the basal expression level of CYP2D6 and contribute to observed interindividual variability of CYP2D6-mediated drug metabolism are not completely understood (Gaedigk et al., 2018). Although considered to be non-inducible by xenobiotics, increased hepatic CYP2D6 expression and activity are observed during pregnancy (Pan et al., 2017) and contribute to increased clearance of drug substrates such as metoprolol, clonidine, dextromethorphan, and paroxetine during pregnancy (Isoherranen and Thummel, 2013).

Prior work has implicated a central role of the farnesoid X receptor (FXR) and downstream transcription factors, including the orphan nuclear receptor small heterodimer partner (SHP), hepatocyte nuclear factor 4 alpha (HNF4α) and liver receptor homologue-1 (LRH-1) in the regulation of CYP2D6 expression (Koh et al., 2014; Pan et al., 2017; Ning et al., 2019). Hepatic FXR activation by bile acids (Choudhuri and Klaassen, 2022) and retinoids (Cai et al., 2010; Koh et al., 2014) induces expression of SHP, which represses the expression of CYP7A1, CYP8B1, and consequently de novo biosynthesis of FXR’s cognate ligands, bile acids. SHP activation also inhibits the activation of HNF4α and LRH-1 and results in downregulation of CYP2D6 in CYP2D6-humanized mice (Koh et al., 2014) and reproduced in human hepatocytes using a synthetic potent FXR agonist, GW4064 (Pan et al., 2015). In contrast, SHP repression, such as during pregnancy, increases HNF4α recruitment to CYP2D6 gene promoter.
resulting in gene induction and increased CYP2D6 metabolic activity in CYP2D6 humanized mice (Koh et al., 2014).

The mechanisms driving CYP2D6 induction during pregnancy remain unclear. Past evidence from in vitro and animal studies supported a mechanism by which declining retinoid concentrations during pregnancy reduced activation of SHP, relieving repression of CYP2D6 expression compared to postpartum (Amaeze et al., 2022). However, recently all-trans-retinoic acid (atRA) plasma concentrations were reported to be higher in pregnancy than postpartum (Amaeze et al., 2022; Czuba et al., 2022; Jeong et al., 2023), and have a positive correlation with the urinary ratio of dextromethorphan / dextrorphan, a marker of CYP2D6 activity (Amaeze et al., 2022). In contrast, in human liver, hepatic atRA concentrations did not associate with CYP2D6 mRNA (Ning et al., 2019). It is unclear if changes in human plasma and/or tissue concentrations of FXR ligands during pregnancy promote reciprocal changes in the endogenous regulation of hepatic SHP expression and its downstream target genes, such as CYP2D6. However, modest changes in bile acid concentrations and/or a shift in bile acid metabolism to favor cholic acid (CYP8B1) over chenodeoxycholic acid (CYP7A1) may occur during healthy pregnancy thereby altering FXR signaling pathways in the liver. Additionally, intestinal bile acid signaling is impaired in human pregnancy, as evident by reduced serum levels of the FXR-regulated hormone, fibroblast growth factor 19 (FGF19) in the third trimester (Ovadia et al., 2019). FGF19 is a critical postprandial regulator of de novo hepatic bile acid synthesis and reduced levels in pregnant women are associated with increased serum concentrations of the bile acid intermediate 7α-hydroxy-4-cholesten-3-one (C4) (Ovadia et al., 2019).

The goal of this study was to characterize the plasma bile acid metabolome in healthy women (n=47) during pregnancy (25-28 weeks gestation) and ≥ 3 months postpartum and to
determine if plasma bile acids correlate with CYP2D6 activity during pregnancy and postpartum. Metabolomics was validated using quantitative liquid-chromatography mass spectrometry analysis of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and the corresponding taurine (T) and glycine (G) conjugates. As the cohort investigated did not have intrahepatic cholestasis (IHC), we hypothesized that during pregnancy plasma bile acids would favor less hydrophobic bile acids (CA vs. CDCA) and positively correlate with CYP2D6 activity similar to that observed in healthy human livers (Ning et al., 2019).
Methods

Materials

Optima LC/MS grade acetonitrile, water, and formic acid were from Thermo Fisher Scientific (Waltham, MA). Human serum (DC Mass Spect Gold MSG 4000) was from Golden West Biologics (Temecula, CA). Cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), and glycodeloxycholic acid (GDCA) were from Sigma Aldrich. The following internal standards were purchased from Steraloids, Inc. (Newport, RI): CA-d₄, CDCA-d₄, DCA-d₄, GCDCA-d₄, GCA-d₄. TCA-d₄, TDCA-d₄, TCDCA-d₄, and GDCA-d₄ were purchased from Cayman Chemicals (Ann Arbor, MI).

Study Participants

Healthy, pregnant women (singleton pregnancies) aged 18-50 years old were enrolled in this study. All enrolled participants were genotyped and only women with CYP2D6 activity scores of 1, 1.5, or 2 participated in the study. All subjects provided written informed consent. Exclusion criteria included a history of obesity (BMI >30 kg/m² based on pre-pregnancy weight), diabetes, kidney disease, liver disease, treatment for mental illness, vitamin A supplementation other than prenatal vitamins, or any current illness involving a fever or cough. The study was approved by the Institutional Review Board at the University of Washington (STUDY00001620, approved 3/28/17) and conducted in accordance with the Declaration of Helsinki principles. This study was registered at ClinicalTrials.gov (NCT03117600).

As part of a larger prospective study evaluating regulators of CYP2D6 activity (Amaeze et al., 2022), each woman completed 3 study days (25-28 weeks gestation (study day 1), 28-32
weeks gestation (study day 2) and ≥ 3 months postpartum (study day 3). Greater than 91% of participants were taking a prenatal vitamin or folic acid supplement during their 25-28 weeks gestation study day. Prenatal vitamins were discontinued between study day 1 and study day 2. During this 3-4 week period between study day 1 and study day 2, each woman took folic acid (1 mg) orally daily. Subjects were randomize using random numbers (1:1) to receive either vitamin A 10,000 IU orally daily or no vitamin A during the period between study day 1 and study day 2. After study day 2, prenatal vitamins were re-started as part of routine clinical care.

**Collection of postprandial plasma samples**

Blood samples were obtained from participants approximately 60 minutes after initiation of breakfast into foil-wrapped, K2 EDTA vacutainer tubes, immediately placed on wet ice, centrifuged within 10 minutes of collection at 3000 g for 10 minutes at 4°C and then plasma was separated and immediately stored at -80°C until bioanalysis. Paired samples from study days 1 and 3 were compared to assess the impact of pregnancy on CYP2D6 activity and plasma bile acids. Samples from study 2 were compared to assess the impact of vitamin A supplementation on plasma bile acids. Samples from all three study days were used to determine the relationship between bile acids and CYP2D6 activity.

**Untargeted metabolomics and pathway analysis**

Metabolomics analysis was carried out by Metabolon Inc., adapted from previously published methods (Collet et al., 2017) and described in detail previously (Enthoven et al., 2023). Bile acid metabolites were identified using automated functions according to a reference library containing the retention time, mass-to-charge ratio, preferred adducts, in-source fragments, and associated spectra for reference standards and confirmed by visual inspection. Peak areas for each bile acid were batch normalized by dividing the raw peak area values by the
median value for that metabolite. Missing values were replaced with the batch-normalized minimum value. MetaboAnalyst 5.0 (http://metaboanalyst.ca) was used to perform joint pathway analysis (Xia and Wishart, 2011; Enthoven et al., 2023).

**Targeted metabolomics and quantification of nine bile acids in human plasma**

Bile acids were quantified from human plasma using methods modified from a previously published (Ning et al., 2019) ultra-high-performance liquid chromatography mass spectrometry (UHPLC-MS/MS) method. A spiked standard curve was prepared in duplicate and four independent quality control (QC) samples were prepared in triplicate in charcoal stripped human serum for the following analytes: CA (9.77–1250 nM; QCs: 25nM, 50 nM, 250nM, 1000 nM), CDCA (19.53–1250 nM; QCs: 50 nM, 250 nM, 415 nM, 1000 nM), DCA (9.77–2500 nM; QCs: 25 nM, 50 nM, 250 nM, 1000 nM), GCDCA (39.06–2500 nM; QCs: 50 nM, 250 nM, 415 nM, 1000 nM), GCA (39.06–2500 nM; QCs: 25 nM, 50 nM, 250 nM, 1000 nM), GDCA (19.53–2500 nM; QCs: 50 nM, 250 nM, 415 nM, 1000 nM), TCA (9.77–2500 nM; QCs: 25 nM, 50 nM, 250 nM, 1000 nM), TDCA (9.77–1250 nM; QCs: 25 nM, 50 nM, 250 nM, 1000 nM), TCDCA (9.77–1250 nM; QCs: 25 nM, 50 nM, 250 nM, 1000 nM). Additionally, non-spiked between day QC samples were prepared using plasma purchased from Bloodworks Northwest (Seattle, WA). Samples outside of the standard curve range were prepared at a 1:10 dilution in blank charcoal stripped serum along with a set of dilution QCs.

For analysis, 60 μL of human plasma samples, standard curve samples, and QC samples were protein precipitated with 120 μL of ice-cold mixture of methanol and acetonitrile (50:50) spiked with 75 nM of the following internal standards: CA-d₄, CDCA-d₄, DCA-d₄, GCDCA-d₄, GCA-d₄, TCA-d₄, TDCA-d₄, TCDCA-d₄, and GDCA-d₄. Samples were gently mixed by pipetting and plates centrifuged at 3,000 g for 40 min at 4° C. The supernatant was transferred to
a new 96-well plate followed by a second centrifugation step at 3,000 g for an additional 30 min at 4°C. One hundred microliters of final cleared supernatant was transferred to a 96-well plate and diluted with 50 µL of LC-MS grade water for UHPLC-MS/MS analysis. All sample preparation was performed on ice.

Plasma bile acids were separated using an Agilent 1290 Infinity I UHPLC (Santa Clara, CA) coupled to a Waters (Milford, MA) ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm). A mobile phase consisting of A) water + 0.1% formic acid and B) acetonitrile + 0.1% formic acid was used at a flow rate of 0.45 mL/min at an initial condition of 25% mobile phase B, held for 2 min before a gradient elution to 40% B by 11 min and 95% by 14.5 min, held at 95% for 2 min before returning to initial conditions.

Bile acids and internal standards detected on an AB Sciex 5500 qTrap Q-LIT mass spectrometer (Foster City, CA operated in negative ion ESI mode. The following transitions were monitored: CA (407.3>407.3), CA-d₄ (411.3>411.3), CDCA (391.3>391.3), CDCA-d₄ (395.3>395.3), DCA (391.2>345.2), DCA-d₄ (395.3>349.3), GCDCA (448.2>73.9), GCDCA-d₄ (452.2>74.2), GCA (454.4>74.1), GCA-d₄ (468.4>73.9), GDCA (448.2>73.9), GDCA-d₄ (452.2>74.2), TCA (452.2>74.2), TCA-d₄ (518.4>79.8), TDCA (502.2>80.0), TDCA-d₄ (502.2>80.0), TCDCA (502.2>80.0), and TCDCA-d₄ (502.2>80.0). Collision energy and delustering potential were optimized for each compound.

Analyte peaks were integrated using MultiQuant 3.1 (Sciex) and peak area ratios were quantified against the standard curve (weighted 1/x). For run acceptance, at each QC concentration, at least 2/3 of QC samples were within 15% of the nominal concentration in accordance with bioanalytical guidelines (FDA and CEDER, 2018).

**Statistical Methods**
Data are expressed as geometric mean and interquartile range (25th percentile, 75th percentile), unless otherwise indicated. Statistical comparisons were performed using GraphPad Prism 9.1 (San Diego, CA). Log-transformed bile acid concentrations from Study Day 1 (SD1) were compared to the paired Study Day 3 (SD3) sample using a two-sided paired T-test. Log-transformed bile acid concentrations from Study Day 2 (SD2) were compared using a two-sided unpaired T-test. P-values for the individual bile acid concentration comparisons were compared for significance at an adjusted alpha of <0.0056 to account for the nine individual bile acid endpoints measured. In addition to reporting individual concentrations of plasma bile acids, surrogate measurements were calculated using published recommendations (Hofmann and Marschall, 2018). Briefly, plasma concentrations of the total plasma bile acid concentration in each participant were approximated through the summation of each of the 9 bile acids. The CA/CDCA ratio was calculated using ‘Total CA’ concentration / ‘Total CDCA’ concentration. Total bile acid concentrations and the CA/CDCA ratio were compared using T-tests and significance measured at p<0.05. Absolute p-values are reported.

To investigate if bile acids are putative regulators of CYP2D6 expression, an exploratory linear mixed-effect regression analysis was performed using the corresponding bile acid plasma concentrations measured at each of the three paired study day windows and previously reported log transformed 4-hour urinary metabolic ratio of dextromethorphan / dextrorphan (Amaeze et al., 2022). All analysis procedures were previously described (Amaeze et al., 2022). All analyses were performed using the NLME package in R 4.1.2 (https://www.r-project.org) (Pinheiro et al., 2022). Bonferroni correction was applied to account for multiple comparisons and absolute p-value reported.
Results

Human Subjects

Forty-seven women with singleton pregnancies completed the study with an average age of 31.9 ± 5.7 years and pre-pregnancy BMI of 26.1 ± 3.3 kg/m². Participants self-identified their race and ethnicity as indicated in Table 1. No participants had any significant alterations in liver or kidney function throughout the study (Table S1). In addition, no participants were diagnosed with intrahepatic cholestasis of pregnancy or pruritis. All other reported medical conditions are as reported in detail previously (Amaeze et al., 2022; Czuba et al., 2022).

There was a higher prevalence of antihistamines, antacids, anti-emetics, and laxatives used during pregnancy compared to postpartum (Table S1). Supplementation with vitamin D, iron, and various forms of omega-3 fatty acids were also reported by some participants during pregnancy and postpartum.

Untargeted plasma metabolomics and bile acid pathway analysis

Previously, exploratory untargeted metabolomic analysis using plasma samples from this cohort identified 708 metabolites significantly different between pregnancy and postpartum and described significant global changes to biochemical pathways including arginine, valine, leucine, isoleucine and xanthine metabolism (Enthoven et al., 2023). Table 2 contains the mean peak area of 34 primary bile acids (unconjugated and taurine (T) and glycine (G) conjugated CA and CDCA) and bile acid secondary metabolites (unconjugated and taurine (T) and glycine (G) conjugated DCA, UDCA, IUDCA, HCA, MCA, etc.). At 25-28 weeks of pregnancy and ≥3 months postpartum, the frequency of a given bile acid being present in the plasma of participants ranged from 51%-100% and 49%-100%, respectively. Based on peak area, 28 of 34 metabolites were significantly different between 25-28 weeks of pregnancy and ≥3 months postpartum.
There was a general trend for higher taurine conjugated metabolite peak areas and lower glycine conjugated metabolites during pregnancy compared to postpartum. A summary of the statistically significant changes identified in the untargeted metabolomics can be found in Figure 1.

Quantitative targeted bile acid metabolomics

To validate the untargeted metabolomic analysis, nine bile acids considered to be the most abundant in plasma (Hofmann and Marschall, 2018) were quantified in plasma samples from 25-28 weeks gestation and ≥3 months postpartum (Table 3). Concentrations of unconjugated bile acids, CA and CDCA were low in plasma compared to the glycine and taurine conjugated bile acids. At 25-28 weeks gestation, geometric mean plasma concentrations of unconjugated CA were 36.6 nM (IQR 18.9 nM; 84.5 nM) and did not differ (p=0.54) from ≥3 months postpartum 42.0 nM (IQR: 22.9 nM; 85.1 nM). In contrast, unconjugated CDCA plasma concentrations were lower (p<0.0001) at 25-28 weeks gestation (40.4 nM (IQR: 17.4 nM; 103.6 nM)) compared to ≥3 months postpartum (126.9 nM (IQR: 51.9 nM; 317.7 nM)). The unconjugated bile acid metabolite, DCA, was also lower (p=0.0043) at 25-28 weeks of gestation (280.0 nM (IQR: 184.4 nM; 480.1 nM) compared to ≥ 3 months postpartum 492.0 nM (IQR 440.1 nM; 825.9 nM).

At 25-28 weeks of gestation, the geometric mean plasma concentrations of the glycine conjugated bile acids, GCDCA and GCA, were 501.1 nM (IQR: 325.6 nM; 916.1 nM) and 386.7 nM (IQR: 193.5 nM; 717.9 nM), respectively. In comparison, at ≥3 months postpartum, GCDCA was higher than during pregnancy (p<0.0001) with a geometric mean concentration of 1,204.0 nM (IQR: 542.1 nM; 2,525.3 nM). In contrast, plasma GCA concentrations were not statistically different (p=0.61) between 25-28 weeks of gestation and ≥3 months postpartum (423.1 nM
(IQR: 222.8 nM; 792.0 nM)). At 25-28 weeks gestation, the concentration of GDCA was 350.1 nM (IQR: 204.6 nM; 761.7 nM) compared to 523.5 nM (IQR: 336.2 nM; 974.6 nM) postpartum, which did not reach statistical significance at the adjusted alpha (p<0.0056).

Bile acids are also conjugated with taurine. Plasma TCA concentrations were higher (p<0.0001) at 25-28 weeks gestation (154.4 nM (IQR: 70.3 nM; 337.0 nM)) than ≥3 months postpartum (47.9 nM (IQR: 18.7 nM; 106.5 nM)). Additionally, TCDCA was higher (p=0.0049) at 25-28 weeks (213.3 nM (IQR:113.2 nM; 514.8 nM)) than ≥3 months postpartum (122.7 nM (IQR: 71.0 nM; 232.1 nM)). Similarly, TDCA concentrations were higher (p<0.0001) at 25-28 weeks (177.0 nM (IQR: 87.6 nM; 464.2 nM)) than ≥3 month postpartum (74.2 nM (30.1 nM; 171.8 nM). Overall, the geometric mean of fold change in the TCA, TDCA, and TCDCA plasma concentrations at 25-28 weeks gestation versus ≥3 month postpartum was 3.2, 2.4, and 1.7, respectively.

It is routine to sum individual bile acid metabolites measured in plasma to approximate the ‘total’ plasma bile acid concentration for comparison to literature data (Hofmann and Marschall, 2018). The sum of the nine bile acids measured was 2,487.2 nM (IQR: 1,458.3 nM; 4,559.7 nM) at 25-28 weeks of gestation and 3,684.9 nM (IQR: 2,038.3 nM; 5,690.4 nM) at ≥3 months postpartum. Although the geometric mean total bile acid pool concentration appeared higher at ≥3 months postpartum (Table 3), this was driven by a few participants with high postprandial bile acid concentrations postpartum. The total postprandial bile acid plasma concentrations were different (p=0.03) at 25-28 weeks gestation when compared to paired plasma samples taken ≥3 months postpartum (Figure 2A). During primary bile acid synthesis, the expression and activity of CYP8B1 determines the ratio of CA to CDCA. Here, the
geometric mean CA/CDCA ratio was 0.78 (IQR: 0.53; 1.0) at 25-28 weeks gestation and 0.36 (IQR: 0.26; 0.52) at ≥3 months postpartum (Figure 2B).

**Impact of vitamin A supplementation on plasma bile acid metabolome**

Vitamin A has been shown to induce the expression of SHP (Cai et al., 2010; Mamoon et al., 2014), a well-known repressor of bile acid synthesis. To determine if vitamin A supplementation impacts postprandial bile acid concentrations, plasma bile acids were measured at 28-32 weeks of gestation and provided in Table S2. No difference was observed (p=0.98) in the total calculated concentration of bile acids between pregnant women who supplemented with low-dose vitamin A (2,344.4 nM; IQR: 1,415.6 nM; 3,889.3 nM) and those without supplementation (2,335.3 nM; IQR: 1,587.0 nM; 3,160.6 nM) (Figure 3A). Likewise, there was no statistical difference observed in the ratio of CA to CDCA between pregnant women receiving vitamin A compared to those not receiving vitamin A (p=0.40) (Figure 3B).

**Bile acids as putative regulators of SHP target gene, CYP2D6**

The relationship between the reported 4-hour urinary DX/DM ratio and the plasma concentration of different bile acids is shown in Figure 4. Taurocholic acid concentrations were correlated with CYP2D6 activity (p=0.0023) across the three study days even after Bonferroni correction for multiple hypothesis testing (9 bile acids and total bile acid, corrected α = 0.005). No other plasma bile acid concentration or calculated total bile acid concentration was observed to have a significant association with CYP2D6 activity.
Discussion

This study reports differences in plasma concentrations of individual bile acids in pregnancy compared to postpartum and provides preliminary evidence an association between plasma TCA concentrations and CYP2D6 regulation. Our results demonstrate that in pregnancy compared to postpartum there is decreased plasma concentrations of unconjugated bile acids and increased plasma concentrations of taurine conjugates. A positive association was observed between plasma TCA concentrations and the 4-hour urinary metabolic ratio of dextrorphan / dextromethorphan. To our knowledge this is the first report to demonstrate that physiological changes to the human plasma bile acid metabolome are positively associated with induction of CYP2D6 activity. However, these findings are supported by a previous report demonstrating that basal CYP2D6 mRNA expression is positively correlated with hepatic total bile acid concentrations, the percentage of hepatic total cholic acid, and expression levels of downstream FXR target genes, SHP, HNF4A and CYP8B1 (Ning et al., 2019). A higher CA to CDCA ratio during pregnancy compared to postpartum was observed both here, and previously (Gagnon et al., 2021) and may suggest an increase in CYP8B1 activity shifting the bile acid metabolic pathway to favor CA formation. The relative potencies of CA, CDCA, and DCA as well as their conjugates towards FXR activation are generally considered comparable in the presence of active transporters (Wang et al., 1999), so the clinical relevance of differential changes between bile acids is currently unclear.

Other transcriptional regulators can modify FXR signaling including retinoids (Cai et al., 2010; Mamoon et al., 2014). Retinoids such as all-trans-retinoic acid induced SHP expression in liver tissue via FXR/RXR signaling pathways (Cai et al., 2010). Retinoids can repress metabolism of bile acids and CYP2D6 mRNA expression and activity (Cai et al., 2010; Stevison
et al., 2019). Here, we demonstrate that vitamin A supplementation during pregnancy does not alter the total concentration of bile acids or any individual bile acids in circulation. Like plasma TCA concentrations, a mild positive association of CYP2D6 activity with atRA plasma concentrations was previously observed in this cohort (Amaeze et al., 2022). Collectively, these findings conflict with the established roles of bile acids and retinoids in the FXR-mediated activation of SHP (Chiang et al., 2000; Watanabe et al., 2004; Cai et al., 2010; Koh et al., 2014), as it would be expected that increased concentrations of either ligand class would promote SHP activity and in turn, repress CYP2D6 expression and activity. Future work is needed to confirm and understand why TCA plasma concentrations, but not TCDCA, TDCA, glycine conjugates, or even total calculated bile acid concentration, is positively correlated with CYP2D6 activity in pregnancy. It may support observations of ligand-specific transcriptional signaling by FXR (Ramos Pittol et al., 2020) or implicate ligand specific differences in the recruitment of coactivator/repressors. Thus, future mechanistic work is required to understand differences in the FXR-SHP signaling patterns of different bile acid metabolites.

A major strength of the study is the validation of the untargeted metabolomic pathway analysis using targeted, quantitative LC-MS/MS methods. The data support prior studies that bile acid homeostasis is altered during human pregnancy (Glantz et al., 2004; Barth et al., 2005; Gagnon et al., 2021; He et al., 2022) and that a subset of normal pregnant women experience asymptomatic hypercholenemia, which is defined by total plasma or serum bile acids > 6 μM (Barth et al., 2005) in the absence of intrahepatic cholestasis (IHC) (He et al., 2022). Total serum bile acids in healthy pregnant women have been shown to be moderately correlated and overlap between the fasting (4.4-14.1 μM) and postprandial (4.7- 20.2 μM) state (Mor et al., 2021). The total bile acid plasma concentrations calculated for the participants in this study were within the
postprandial range for normal pregnancies (Mor et al., 2021) and no participants were diagnosed with pruritus or IHC during the study. Bile acid conjugation to taurine or glycine results in bile acids that are less hydrophobic and carry less risk of liver toxicity as concentrations rise compared to unconjugated bile acids (Hofmann, 1984). Collectively, when comparing pregnancy and postpartum plasma concentrations, there was an increase in taurine conjugated bile acids. The enzyme responsible for conjugation, bile acid-CoA:amino acid N-acyltransferase (BAAT), has similar enzyme activity towards glycine or taurine, so differences in conjugation are likely driven by amino acid availability (Styles et al., 2016). Glycine is needed for production of heme and glutathione and can be obtained dietarily or biosynthesized in humans. Recently, it has been established that there is an indispensable maternal need for dietary glycine in late gestation (30-40 weeks) (Rasmussen et al., 2021). It is possible that there is a conditional switch to bile acid taurine conjugation earlier in pregnancy to compensate for increased metabolic demand for glycine. Thus, measured differences in the plasma concentration of individual bile acids likely represents collective changes to the biosynthesis pathway, taurine or glycine hepatic availability and potentially hepatic bile acid uptake in human pregnancy. A limitation of the current analysis is that some participants reported use of supplements (e.g., vitamin D; omega-3s/fish oils) that can indirectly alter bile acid homeostasis by altering bile acid metabolism and feedback mechanisms (Jacobs et al., 2016; Cieślak et al., 2018). The study was not designed to investigate these interactions or differences related to pregnancy complications. In addition, the bile acid precursor 7α-hydroxy-4-cholesten-3-one was not measured, limiting interpretation as to whether increases in the taurine conjugates were associated with increased bile acid synthesis (Sauter et al., 1996; Ovadia et al., 2019) or other metabolic and transport processes (Ovadia et al., 2019). Although prior work has shown that pregnancy is associated with reduced enterohepatic
regulation of bile acid de novo synthesis (Ovadia et al., 2019), future work is needed to establish the role of alternative mechanisms promoting altered bile acid homeostasis in pregnancy. Collectively, this study supports that bile acid homeostasis is altered in human pregnancy. The results are vital to understanding transient changes to bile acid homeostasis in pregnancy and will provide preliminary insight into drivers of pregnancy-related changes in CYP2D6-mediated drug metabolism in humans.
Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author upon reasonable request.

Author Contributions

Participated in research design: Hebert and Isoherranen

Conducted experiments: Czuba, Malhotra, Enthoven, Fay, Moreni, Mao, Shi, Huang, and Hebert

Contributed new reagents/analytical tools: Isoherranen, Totah, Hebert

Performed data analysis: Czuba, Malhotra, Enthoven, Shi, Huang, Hebert, Totah, and Isoherranen

Wrote or contributed to the writing the manuscript: Czuba, Malhotra, Enthoven, Fay, Moreni, Mao, Shi, Huang, Totah,Isoherranen, and Hebert
References


Footnotes

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Figure legends

**Figure 1**: Pathway for synthesis of primary (marked in purple) and secondary (marked in grey) bile acids constructed using the mean peak areas measured in the untargeted metabolomics assays and constructed based on KEGG pathways for bile acid synthesis. An alternate pathway is also depicted (marked in red). Downward pointing arrows indicate lower metabolite peak areas in pregnancy (25-28 weeks of gestation) compared to ≥3 months postpartum, (marked in red), while upward arrows indicate higher peak area during pregnancy compared to postpartum (marked in green). Double pointed horizontal arrows indicate no significant change (blue). Tauro-β-muricholic acid and glyco-β-muricholic acid were not included in the pathway as they are not recognized as major bile acid metabolites in humans.

**Figure 2**: Secondary comparisons of A) total bile acids and B) total CA to CDCA ratio which are calculated from the nine bile acids measured using a targeted LC-MS/MS method and represented as the individual values or geometric mean ± 95% CI, respectively. Plasma samples from n=47 women drawn in pregnancy (25-28 weeks) and postpartum (≥3 months) were used in the analysis. Log-transformed concentrations measured at 25-28 weeks of gestation and ≥3 months postpartum were compared using two-way paired t-tests without correction for multiple comparisons. Absolute p-values are reported.

**Figure 3**: Comparison of the A) calculated total bile acid concentration and B) calculated total CA to CDCA ratio plasma concentration between pregnant women at 28-32 weeks of gestation (Study Day 2) with vitamin A supplementation (n=24) or without (n=23). Calculations are based on the quantification of nine bile acids measured using a targeted LC-MS/MS method and
represented as geometric mean ± 95% CI. Log-transformed concentrations measured were compared using two-way unpaired t-tests without correction for multiple comparisons. Absolute p-values are reported.

**Figure 4:** Log-transformed urinary dextrorphan (DX)/dextromethorphan (DM) ratio versus plasma A) cholic acid, B) chenodeoxycholic acid, C) deoxycholic acid, D) taurocholic acid, E) taurochenodeoxycholic acid, F) taurodeoxycholic acid, G) glycocholic acid, H) glycochenodeoxycholic acid, I) glycodeoxycholic acid, and J) total bile acid concentrations (nM). Light-blue solid dots represent repeated measurements from n=47 participants, while each dark-blue solid line represents the prediction from a linear mixed-effect model for each individual participant. The p-value of the regression coefficient is displayed.
Tables

Table 1: Participants’ weight (mean ± SD) and self-reported demographics.

<table>
<thead>
<tr>
<th>Pregnant Women (N=47)</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>31.9 ± 5.7</td>
</tr>
<tr>
<td>Pre-pregnancy BMI</td>
<td>26.1 ± 3.3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.4 ± 10.4</td>
</tr>
</tbody>
</table>

**Race**

<table>
<thead>
<tr>
<th>Race</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
<td>35 (74.5%)</td>
</tr>
<tr>
<td>Black</td>
<td>4 (8.5%)</td>
</tr>
<tr>
<td>Asian</td>
<td>5 (10.6%)</td>
</tr>
<tr>
<td>Pacific Islander</td>
<td>1 (2.1%)</td>
</tr>
<tr>
<td>Caucasian and Asian</td>
<td>2 (4.3%)</td>
</tr>
</tbody>
</table>

*1 participant self-identified as Caucasian of Hispanic descent and all 4 participants identifying as Black were of African descent.*
Table 2: Batch normalized peak areas of bile acids measured using untargeted metabolomic methods. Plasma samples from n=47 women drawn in pregnancy (25-28 weeks) and postpartum (≥3 months) were used in the analysis. The paired Pregnancy/Postpartum ratio was calculated for each participant and presented as the geometric mean and interquartile range). Paired Pregnancy/Postpartum ratio p-values are calculated from paired t-tests, and pathway p-values (q-values) are adjusted by pathway-weighting and the false discovery rate (FDR). Pregnancy/Postpartum significant changes denoted with * and absolute p-values are reported.

<table>
<thead>
<tr>
<th>Bile Acid Metabolite</th>
<th>Pregnant Mean ± SD</th>
<th>Postpartum Mean ± SD</th>
<th>Paired Pregnancy/Postpartum Ratio Geom. Mean (25th; 75th)</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholate</td>
<td>2.13 ± 2.84</td>
<td>1.72 ± 1.63</td>
<td>0.96 (0.53 ; 1.97)</td>
<td>0.8</td>
<td>0.081</td>
</tr>
<tr>
<td>Glycocholate</td>
<td>1.53 ± 1.44</td>
<td>1.90 ± 2.54</td>
<td>0.91 (0.43 ; 1.69)</td>
<td>0.6</td>
<td>0.0619</td>
</tr>
<tr>
<td>Taurocholate</td>
<td>3.35 ± 3.80</td>
<td>1.09 ± 1.26</td>
<td>3.72 (1.40 ; 7.49)</td>
<td>8.50E-07</td>
<td>2.22E-07</td>
</tr>
<tr>
<td>Chenodeoxycholate</td>
<td>0.75 ± 0.83</td>
<td>1.32 ± 2.50</td>
<td>0.77 (0.43 ; 1.06)</td>
<td>0.08</td>
<td>0.0105</td>
</tr>
<tr>
<td>Glycochenodeoxycholate</td>
<td>0.88 ± 0.69</td>
<td>2.23 ± 2.16</td>
<td>0.41 (0.21 ; 0.80)</td>
<td>4.20E-07</td>
<td>1.14E-07</td>
</tr>
<tr>
<td>Taurochenodeoxycholate</td>
<td>2.07 ± 1.87</td>
<td>1.33 ± 1.54</td>
<td>1.77 (0.79 ; 3.87)</td>
<td>0.005</td>
<td>0.0008</td>
</tr>
<tr>
<td>Tauro-beta-muricholate</td>
<td>2.76 ± 4.09</td>
<td>0.42 ± 0.55</td>
<td>7.59 (3.80 ; 15.70)</td>
<td>2.30E-18</td>
<td>3.36E-18</td>
</tr>
<tr>
<td>Glyco-beta-muricholate</td>
<td>1.62 ± 1.21</td>
<td>0.96 ± 0.76</td>
<td>1.65 (0.96 ; 2.88)</td>
<td>0.0002</td>
<td>3.77E-05</td>
</tr>
<tr>
<td>Glycochenodeoxycholate glucuronide</td>
<td>2.42 ± 2.04</td>
<td>0.78 ± 0.73</td>
<td>3.08 (2.20 ; 4.62)</td>
<td>1.49E-14</td>
<td>1.11E-14</td>
</tr>
<tr>
<td>Glycochenodeoxycholate 3-sulfate</td>
<td>1.18 ± 1.84</td>
<td>1.29 ± 0.75</td>
<td>0.67 (0.33 ; 1.02)</td>
<td>0.003</td>
<td>0.0004</td>
</tr>
<tr>
<td>Glycocholate glucuronide</td>
<td>1.13 ± 0.97</td>
<td>0.61 ± 0.75</td>
<td>1.82 (0.97 ; 3.83)</td>
<td>0.0001</td>
<td>1.71E-05</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>0.85 ± 0.69</td>
<td>1.33 ± 0.79</td>
<td>0.59 (0.31 ; 1.01)</td>
<td>0.002</td>
<td>0.0003</td>
</tr>
<tr>
<td>Deoxycholic acid 12-sulfate</td>
<td>1.13 ± 0.81</td>
<td>1.51 ± 1.40</td>
<td>0.78 (0.42 ; 1.35)</td>
<td>0.1</td>
<td>0.0135</td>
</tr>
<tr>
<td>Deoxycholic acid glucuronide</td>
<td>0.84 ± 0.71</td>
<td>1.44 ± 1.16</td>
<td>0.56 (0.37 ; 0.92)</td>
<td>4.30E-06</td>
<td>1.01E-06</td>
</tr>
<tr>
<td>Glycodeoxycholate</td>
<td>1.15 ± 1.10</td>
<td>1.76 ± 1.88</td>
<td>0.65 (0.33 ; 1.36)</td>
<td>0.02</td>
<td>0.0032</td>
</tr>
<tr>
<td>Taurodeoxycholate</td>
<td>1.95 ± 1.86</td>
<td>0.89 ± 0.84</td>
<td>2.18 (0.91 ; 4.73)</td>
<td>0.0003</td>
<td>5.15E-05</td>
</tr>
<tr>
<td>Taurodeoxycholic acid 3-sulfate</td>
<td>2.89 ± 3.35</td>
<td>0.51 ± 0.43</td>
<td>5.74 (3.03 ; 11.61)</td>
<td>2.10E-16</td>
<td>2.31E-16</td>
</tr>
<tr>
<td>Lithocholate sulfate</td>
<td>0.75 ± 0.68</td>
<td>1.72 ± 1.15</td>
<td>0.40 (0.22 ; 0.70)</td>
<td>3.30E-07</td>
<td>8.98E-08</td>
</tr>
<tr>
<td>Glycolithocholate</td>
<td>0.52 ± 0.52</td>
<td>1.63 ± 0.97</td>
<td>0.26 (0.11 ; 0.55)</td>
<td>1.00E-08</td>
<td>3.38E-09</td>
</tr>
<tr>
<td>Glycolithocholate sulfate</td>
<td>0.77 ± 0.42</td>
<td>1.59 ± 0.95</td>
<td>0.54 (0.32 ; 0.75)</td>
<td>0.0001</td>
<td>2.54E-05</td>
</tr>
<tr>
<td>Taurolithocholate 3-sulfate</td>
<td>1.49 ± 0.99</td>
<td>1.01 ± 0.89</td>
<td>1.83 (0.99 ; 2.99)</td>
<td>4.10E-05</td>
<td>8.86E-06</td>
</tr>
<tr>
<td>Ursodeoxycholate</td>
<td>0.68 ± 0.95</td>
<td>1.85 ± 4.36</td>
<td>0.43 (0.26 ; 0.99)</td>
<td>0.0001</td>
<td>1.96E-05</td>
</tr>
<tr>
<td>Compound</td>
<td>Mean ± SD</td>
<td>Median ± IQR</td>
<td>P</td>
<td>Bonferroni Adjusted P</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------</td>
<td>--------------</td>
<td>---</td>
<td>----------------------</td>
<td></td>
</tr>
<tr>
<td>Isoursodeoxycholate</td>
<td>0.89 ± 1.34</td>
<td>3.73 ± 5.54</td>
<td>0.25 (0.12 ; 0.48) *</td>
<td>9.00E-15</td>
<td></td>
</tr>
<tr>
<td>Glycoursodeoxycholate</td>
<td>0.99 ± 1.70</td>
<td>3.18 ± 4.59</td>
<td>0.30 (0.15 ; 0.58) *</td>
<td>6.00E-11</td>
<td></td>
</tr>
<tr>
<td>Glycoursodeoxycholic acid sulfate</td>
<td>1.75 ± 2.83</td>
<td>1.24 ± 1.16</td>
<td>0.90 (0.47 ; 1.62)</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Tauroursodeoxycholate</td>
<td>1.07 ± 1.62</td>
<td>1.21 ± 2.77</td>
<td>1.12 (0.66 ; 2.01) *</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Hyocholate</td>
<td>1.02 ± 0.99</td>
<td>1.25 ± 0.94</td>
<td>0.72 (0.35 ; 1.29) *</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Glycohyocholate</td>
<td>1.10 ± 0.89</td>
<td>1.44 ± 1.13</td>
<td>0.78 (0.49 ; 1.04) *</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Glycocholenate sulfate</td>
<td>0.82 ± 0.43</td>
<td>1.37 ± 0.59</td>
<td>0.57 (0.39 ; 0.81) *</td>
<td>2.10E-09</td>
<td></td>
</tr>
<tr>
<td>Taurocholenate sulfate</td>
<td>1.67 ± 0.98</td>
<td>0.82 ± 0.54</td>
<td>2.10 (1.34 ; 2.88) *</td>
<td>6.70E-10</td>
<td></td>
</tr>
<tr>
<td>3b-hydroxy-5-cholenoic acid</td>
<td>0.66 ± 0.49</td>
<td>1.17 ± 0.63</td>
<td>0.53 (0.35 ; 0.80) *</td>
<td>3.60E-09</td>
<td></td>
</tr>
<tr>
<td>Glycodeoxycholate 3-sulfate</td>
<td>1.66 ± 1.60</td>
<td>1.02 ± 0.81</td>
<td>1.61 (0.84 ; 2.54) *</td>
<td>0.0014</td>
<td></td>
</tr>
<tr>
<td>Taurochenodeoxycholic acid 3-sulfate</td>
<td>2.41 ± 3.58</td>
<td>0.78 ± 0.65</td>
<td>2.55 (1.31 ; 4.40) *</td>
<td>2.20E-07</td>
<td></td>
</tr>
<tr>
<td>Glycodeoxycholate glucuronide</td>
<td>2.08 ± 2.02</td>
<td>0.59 ± 0.69</td>
<td>3.77 (2.00 ; 5.72) *</td>
<td>2.30E-12</td>
<td></td>
</tr>
</tbody>
</table>

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Table 3: Plasma concentrations of nine bile acids measured using targeted LC-MS/MS methods. Plasma samples from n=47 women drawn in pregnancy (25-28 weeks) and postpartum (≥3 months) were used in the analysis. Log-transformed concentrations measured at 25-28 weeks of gestation and ≥3 months postpartum were compared using two-way paired t-tests. Significance was measured against an adjusted alpha of p<0.0056 to account for the nine separate comparisons. Absolute p-values are reported, and significant differences indicated in bold font. The sum of all nine bile acids was calculated for each participant and geometric mean and IQR reported, along with percentage of unconjugated bile acids (CA, CDCA, DCA), taurine conjugates (TCA, TCDCA, TDCA), and glycine conjugates (GCA, GCDCA, GDCA). For each bile acid, the pregnancy to postpartum ratio was calculated for each participant and the geometric mean ratio and IQR reported.
<table>
<thead>
<tr>
<th>Bile Acids</th>
<th>25-28 weeks gestation (nM)</th>
<th>25-28 weeks gestation (Interquartile range 25th; 75th)</th>
<th>≥3 months postpartum (nM)</th>
<th>≥3 months postpartum (Interquartile range 25th; 75th)</th>
<th>Paired Ratio</th>
<th>Pregnancy/Postpartum adjusted alpha p&lt;0.0056</th>
<th>P-value A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholic acid</td>
<td>36.6</td>
<td>22.9; 85.1</td>
<td>0.9</td>
<td>(0.4; 0.8)</td>
<td></td>
<td></td>
<td>0.54</td>
</tr>
<tr>
<td>Chenodeoxycholic acid</td>
<td>40.4</td>
<td>126.9</td>
<td>0.3</td>
<td>(0.1; 0.3)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Deoxycholic acid</td>
<td>280.0</td>
<td>492.0</td>
<td>0.6</td>
<td>(0.3; 0.6)</td>
<td></td>
<td></td>
<td>0.0043</td>
</tr>
<tr>
<td>Glycocholic acid</td>
<td>386.7</td>
<td>222.8; 792.0</td>
<td>0.9</td>
<td>(0.4; 1.0)</td>
<td></td>
<td></td>
<td>0.61</td>
</tr>
<tr>
<td>Glycochenodeoxycholic acid</td>
<td>501.1</td>
<td>542.1; 2,525.3</td>
<td>0.4</td>
<td>(0.2; 0.5)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glycodeoxycholic acid</td>
<td>350.1</td>
<td>336.2; 974.6</td>
<td>0.7</td>
<td>(0.4; 0.7)</td>
<td></td>
<td></td>
<td>0.047</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>154.4</td>
<td>18.7; 106.5</td>
<td>3.2</td>
<td>(1.2; 3.0)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Taurochenodeoxycholic acid</td>
<td>213.3</td>
<td>71.0; 232.1</td>
<td>1.7</td>
<td>(0.8; 1.6)</td>
<td></td>
<td></td>
<td>0.0049</td>
</tr>
<tr>
<td>Taurodeoxycholic acid</td>
<td>177.0</td>
<td>30.1; 171.8</td>
<td>2.4</td>
<td>(1.2; 2.2)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Σ Bile acids

% Unconjugated bile acids

% Glycine conjugated bile acids

% Taurine conjugated bile acids

2,487.2 (1,458.3; 4,559.7) 3,684.9 (2,038.3; 5,690.4) 15.9% (11.0%; 31.6%) 22.5% (13.5%; 40.2%) 53.0% (46.9%; 61.3%) 63.3% (53.3%; 74.7%) 23.3% (19.0%; 35.1%) 7.2% (5.1%; 12.3%)
Log-transformed data compared using a two-way paired t-test. Significance was determined using an adjusted alpha = 0.0056 and denoted by bold font.
Figure 2

A. Total Bile Acid [nM]

- 25-28 weeks
- ≥ 3 months PP

- 0
- 10000
- 20000
- 30000

- p<0.004

B. Total CA to CDCA Ratio

- 25-28 weeks
- ≥ 3 months PP

- 0.0
- 0.5
- 1.0
- 1.5
- 2.0
- 2.5

- <0.0001
Figure 3
Figure 4