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Urinary Bile Acids Profile of Cesarean Sectioned Newborns is Characterized by Oxidative Metabolism of Primary Bile Acids: Limited Roles of Fetal-specific CYP3A7 in Cholate Oxidations.

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Oxidative Metabolism of Primary Bile Acids in Newborns

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Abbreviations

3-dehydroCA, 3-oxo, 7α, 12α-dihydroxy-5β-cholan-24-oic acid

3-dehydroDCA, 3-oxo, 12α-hydroxy-5β-cholan-24-oic acid

12-epiCA, 3α, 7α, 12β-trihydroxy-5β-cholan-24-oic acid

12-epiDCA, 3α, 12β-dihydroxy-5β-cholan-24-oic acid

12-oxoCDCA, 3α, 12-oxo-hydroxy-5β-cholan-24-oic acid

12-oxoLCA, 3α, 12-oxo-hydroxy-5β-cholan-24-oic acid

αHCA, 3α, 6α, 7α-trihydroxy-5β-cholan-24-oic acid

BA, bile acid

CA, cholate, 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid

CDCA, chenodeoxycholate, 3α, 7α-dihydroxy-5β-cholan-24-oic acid

CDCA-1β-ol, 1β, 3α, 7α-dihydroxy-5β-cholan-24-oic acid

CL_{int}, intrinsic clearance

DCA, deoxycholate, 3α, 12α-dihydroxy-5β-cholan-24-oic acid

DCA-1β-ol, 1β, 3α,12α-trihydroxy-5β-cholan-24-oic acid

DCA-4β-ol, 3α, 4β, 12α-trihydroxy-5β-cholan-24-oic acid

DCA-5β-ol, 3α, 5β, 12α-trihydroxy-5β-cholan-24-oic acid

DCA-6α-ol, 3α, 6α, 12α-trihydroxy-5β-cholan-24-oic acid

DCA-6β-ol, 3α, 6β, 12α-trihydroxy-5β-cholan-24-oic acid

DCA-19-ol, 3α, 12α, 19-trihydroxy-5β-cholan-24-oic acid

DMSO, dimethylsulfoxide

GCA, glycocholate

GDCA, glycodeoxycholate

HDCA, hyodeoxycholate, 3α, 6α-dihydroxy-5β-cholan-24-oic acid

HLM: human liver microsomes

HLM-IF: infant liver microsomes

isoCA, 3β, 7α, 12α-trihydroxy-5β-cholan-24-oic acid

LCA, lithocholate, 3α-hydroxy-5β-cholan-24-oic acid

LC-MS/MS, liquid chromatography with tandem mass spectrometry

OPLS-DA, orthogonal projection to latent structures

P450, cytochrome P450

PCA, Principal Component Analysis

TCA, taurocholate

TDCA, taurodeoxycholate

Tetra-BA: tetrahydroxy-cholan-24-oic acid

UCA, ursocholate, 3α, 7β, 12α-trihydroxy-5β-cholan-24-oic acid

UDCA, 3α, 7β-dihydroxy-5β-cholan-24-oic acid

Abstract

This work aims to investigate how the bile acid metabolism of newborns differs from that of adults along the axis of primary, secondary and tertiary bile acids (BAs). The total unconjugated BA profiles were quantitatively determined by enzyme-digestion techniques in urine of 21 cesarean sectioned newborns, 29 healthy parturient women, 30 healthy male and 28 healthy non-pregmant female. Due to lack of developed gut microbiota, newborns exhibited a poor metabolism of secondary BAs as expected. Accordingly, the tertiary BAs contributed limitedly to the urinary excretion of BAs in newborns despite that their tertiary-to-secondary ratios significantly increased. As a result, the primary BAs of newborns underwent extensive oxidation metabolism, resulting in elevated urinary levels of some fetal-specific BAs, including 3-dehydroCA, isoCA, 12-oxoCDCA and nine tetrahydroxy-cholan-24-oic acids (Tetra-BAs). Parturient women had significantly elevated urinary levels of tertiary BAs and fetal-specific BAs compared to female control, indicating that they may be excreted into amniotic fluid for maternal disposition. *In vitro* metabolism assay in infant liver microsomes showed that four Tetra-BAs and 3-dehydroCA were hydroxylated metabolites of cholate, glycocholate and particularly taurocholate. However, the recombinant P450 enzyme assay found that the fetal specific CYP3A7 did not contribute to these oxidation metabolisms as much as expected compared to CYP3A4. In conclusion, newborns show the BA metabolism pattern predominated by primary BA oxidations due to immaturity of secondary BA metabolism. Translational studies following this finding may bring new ideas and strategies for both pediatric pharmacology and diagnosis and treatment of perinatal cholestasis associated diseases.

Significance Statement

The prenatal BA disposition is different from adults due to lack of gut microbiota. However, it's remains poorly defined how the BA metabolism of newborns differs from that of adults along the axis of primary, secondary and tertiary BAs. This works demonstrated that the urinary BA profiles of cesarean sectioned newborns are characterized by oxidative metabolism of primary BAs, in which the fetal-specific CYP3A7 plays a limited role in the downstream oxidation metabolism of cholate.

Introduction

Bile acids (BAs) are amphipathic steroid metabolites functioning not only as agents facilitating the solubilization and absorption of lipids, but also as signal molecules mediating crosstalk between host and gut microbiota associated with metabolism and immunity. BAs are produced from cholesterol almost exclusively in the liver and subsequently undergo extensive enterohepatic circulation driven by a series of host-gut microbial metabolism and transport mechanisms. Fundamental mechanisms for the synthesis of primary BAs in host liver and the metabolism of secondary BAs by gut microbes had been well defined in adults population in the last century (Russell, 2003; Halilbasic et al., 2013; Dawson and Karpen, 2015). Recent works demonstrated that human cytochrome P450 (CYP) 3A enzymes are exclusively responsible for the modification of secondary BAs into tertiary BAs, which has defined the axis of primary (host liver) - secondary (gut microbiota) - tertiary (host liver) BAs in adults (Chen et al., 2019; Zhang et al., 2019). In the intestinal tract of fetus, however, there is a limited colonization of bacteria responsible for secondary BA production. On the other hand, CYP3A7 rather than CYP3A4 is the major CYP3A isoform expressed in the fetal and neonatal livers (Schuetz et al., 1993; Kivisto et al., 1996). Therefore, the prenatal BA disposition mechanism of fetus is different from that of the adults due to a lack of secondary BAs production and the downstream tertiary BA metabolism.

Difference of BA metabolism between adults and fetus has been extensively evidenced by early gas chromatography coupled with mass spectrometry (GC-MS) based studies, in which a series of BA metabolites with "unusual" oxidation sites, including C-1β, -2β, -4β, -6α/β and -19, were detected and identified in early human life (Sjövall et al., 2010). Most of these studies employed chemical and/or enzymatic hydrolysis techniques to detect all BAs in their unconjugated forms, because it's rather challenging to incorporate these many unconjugated, N-amidated, sulfated and/or glucuronidated BAs in single bioanalytical panel. The development-related BA metabolites may be formed from cholate (CA), chenodeoxycholate (CDCA), deoxycholate (DCA) and lithocholate (LCA). Some of them, particularly the hydroxylated metabolites of CA, CDCA and DCA, were reported to have significantly higher levels in amniotic fluid, urine and feces of pregnant women, neonatal urine, gastric contents and feces (Alme and Sjovall, 1980; Tohma et al., 1985; Tohma et al., 1986; Mahara et al., 1987; Setchell et al., 1988; Nakagawa and Setchell, 1990; Ikegawa et al., 1994; Kurosawa et al., 1995; Kimura et al., 1997; Meng et al., 1997b; Suzuki et al., 1997), and were also induced in patients with cholestasis, liver cirrhosis and other liver diseases

compared to healthy adults (Shoda et al., 1988; Batta et al., 1989; Shoda et al., 1989; Miyara et al., 1990; Shoda et al., 1990; Meng et al., 1997a; Kakiyama et al., 2014). Interestingly, these oxidation sites, in addition to C-5β, have been recently characterized as CYP3A4 and CYP3A7 catalyzed tertiary oxidation sites of DCA, glycodeoxycholate (GDCA) and taurodeoxycholate (TDCA) (Zhang et al., 2019). CYP3A7 favors the oxidations at C-19, C-4β, C-6α, C-3β and C-1β, whereas CYP3A4 favors the oxidations at C-5β and C-6β compared to each other (Chen et al., 2019). These tertiary metabolites except for DCA-19-ol were detected in serum and predominantly in urine of healthy adults (Zhu et al., 2018; Zhang et al., 2019).

During the past decades, the BA profiling techniques have shifted from GC-MS to liquid chromatography coupled with mass spectrometry (LC-MS) (Zhu et al., 2018). As far as our knowledge, more than ten years have passed since the latest GC-MS based research focusing on the prenatal BA metabolism (Kimura et al., 1997; Maeda et al., 2003; Kumagai et al., 2007; Nishiura et al., 2010). There have been no reports on the perinatal BA metabolism using LC-MS techniques probably because commercial sources of the authentic standards are extremely limited. Currently, it is still unclear how the fetal BA metabolism is different from that of adults along the axis of primary, secondary and tertiary BAs. This work therefore comprehensively performed LC-MS based BA profiling of urine samples from cesarean sectioned newborns, parturient women and healthy adults. The validated enzyme-digestion based LC-MS method was used to highlight the redox metabolism and omit the conjugation metabolism of BAs. It was confirmed that the metabolism of secondary and tertiary BAs did not contribute significantly to the BA metabolism phenotype of newborns. As a result, the urinary BA profiles of newborns are characterized by oxidative metabolism of primary BAs, which might have played a vital role in BA elimination in early life. Beyond our expectations, however, preliminary *in vitro* evidences showed that the fetal-specific CYP3A7 played a limited role in the downstream oxidation metabolism of CA.

Materials and Methods

Materials and Reagents

Authentic standards of 31 unconjugated BAs and 4 stable isotope-labeled internal standards were obtained from Steraloids (Newport, RI, USA), TRC (Toronto, Canada), Santa Cruz (Dallas, TX, USA) or Sigma-Aldrich (St. Louis, MO, USA) as previously described (Zhu et al., 2018). Six DCA metabolites, DCA-1β-ol, DCA-4β-ol, DCA-5β-ol, DCA-6β-ol, DCA-6α-ol and DCA-19-ol, were synthesized as described in our recent report (Zhang et al.,

2019). Sulfatase from Helix pomatia Type H-1, β-glucuronidase from Helix pomatia Type H-1 and choloylglycine hydrolase from clostridium perfringens were purchased from Sigma-Aldrich. Urinary creatinine colorimetric assay kit was purchased from Cayman Chemical (MI, USA). Fourteen single-donor adult liver microsomes were obtained from BD Bioscience (Woburn, MA, USA). Eight single-donor infant liver microsomes were purchased from Corning (Corning, NY, USA). The donor information was summarized in Supplemental **Table S1**. Human liver microsomes from 150 (76 female and 74 male) mixed gender pooled donors were purchased from Corning (Tewksbury, MA, USA). The recombinant human CYP3A4, CYP3A5, CYP3A7 enzymes prepared from baculovirus-transfected insect cells (Supersomes™), NADPH regenerating system solution A (NADPH-A, containing 26 mM $NADP^+$, 66 mM glucose-6-phosphate and 66 mM $MgCl_2$ in H_2O), NADPH regenerating system solution B (NADPH-B, containing 40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate) and 0.5 M pH7.4 phosphate buffer (PBS) were purchased from Corning. Calcium chloride ($CaCl_2$, 96%) was purchased from Tianjin Kemiou Chemical (China). LC-MS grade methanol, acetonitrile and formic acid were purchased from Sigma-Aldrich. DMSO was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ultra-pure water was obtained by using a Milli-Q system (Millipore, Bedford).

Human Urine Samples

Overnight fasting urine samples were collected at Institute of Clinical Pharmacology, West China Hospital of Sichuan University from 58 healthy volunteers including 30 males and 28 females. All urine samples were stored at -80°C until analysis. Briefly, the inclusion criteria for healthy subjects were normal blood, liver and kidney functions; negative test results for the biomarker of infectious diseases including hepatitis B, hepatitis C, HIV and Treponema pallidum; no abnormalities in electrocardiogram, abdominal ultrasonography and chest radiography; no history of gastrointestinal surgery except for appendicectomy; and no ingestion of any medications or dietary supplements two-weeks before urine collections. Pregnancy was excluded for all females by serum HCG test. The demographic information of healthy adult population enrolled in this work was listed in supplementary **Table S2**. The studies of BAs in healthy adult urine samples were approved by the Institutional Review Board of West China Hospital of Sichuan University.

Twenty-nine healthy parturient women were recruited at the Labor and Delivery Room of West China Second University Hospital of Sichuan University. Medicinal history of routine prenatal examinations was checked to exclude those with abnormalities during pregnancy, including gestational diabetes mellitus, intrahepatic cholestasis of pregnancy,

gestational hypertension, iron deficiency anemia during pregnancy, etc. Urine samples of the parturient women were collected from catheterization before caesarean section. All neonates were delivered by caesarean section and their diaper disposals were collected as much as possible within 48 hours of birth. Diaper samples without contamination of meconium were collected from twenty-one newborns, stored in ice box and transferred to the bioanalytical lab. The urine samples of newborns that were absorbed by diaper gel were isolated with the protocol described below. All urine samples were stored at -80°C until analysis. The demographic information of pregnant and newborns enrolled in this work were listed in supplementary **Table S2** and **Table S3**. The studies of BAs in urine samples of pregnant and newborns were approved by the Institutional Review Board of Sichuan University.

Validation of Diaper Urine Isolation Protocol

We have tested two methods to isolate urine samples that were absorbed by diaper gel. The methanol method was to process the diaper gel with 5 folds (v/w) of methanol by overnight sonication. The $CaCl_2$ method was to process the gel with 2% (w/w) $CaCl_2$ according to literatures (Hu et al., 2004; Liu et al., 2014). The BAs extracted from diaper gel were determined with the enzyme digestion method described below and the recoveries were calculated in comparison to the direct analysis of a pooled urine sample of in-lab volunteers. As shown in supplementary **Table S4**, extraction recoveries of the time-consuming methanol method for most BAs were below 50%. However, the data of $CaCl_2$ method were within 80-110% and remained stable during multiple tests except for the most hydropholic LCA (64±9%, n=4). Additional test of creatinine confirmed the $CaCl_2$ method may extract urinary creatinine from diaper gel with recoveries of 95±4% (n=8). The $CaCl_2$ method was therefore employed in this work. Briefly, the diaper urine was released and separated by adding $CaCl_2$ (2%, w/w) into the wet gel, vortex mixed at 1500 rpm for 10 min and centrifuged at 10°C at 600 g for 10 min.

Urinary Creatinine Assay

Urinary creatinine levels were measured by the alkaline picrate method with kits purchased from Cayman Chemical (MI, USA). The absorbance value at 490 nm was measured before and after acid addition and the difference between the absorbance was used to calculate the creatinine levels according to the creatinine standard curve.

Sample Preparation for Unconjugated BA Profiling

Analysis of BA profiles was performed using the enzyme digestion techniques published in our recent work (Zhu et al., 2018). Following the strategy employed by the GC-MS based BA analysis, this method was designed and validated to detect all BAs in their unconjugated

forms. Information of the N-amidated, sulfated and/or glucuronidated of BAs was accordingly lost in this work. First, 50 µL urine sample was transferred to 700 µL round well 96-well plate. Second, 150 µL sodium acetate buffers (pH 5.0) containing 100 U of choloylglycine hydrolase, 50 U of sulfatase and 500 U of β-glucuronidase were added to hydrolyse the N-acylamidated, glucuronidated and/or sulfated forms into the unconjugated forms. The calibration and quality control samples were prepared with 150 µL buffers without any enzymes. The plate was incubated at 37°C for 6 hr and subsequently lyophilized. 200 uL acetonitrile containing 1% formic acid and 100 nM internal standards (LCA-2,2,4,4-D4, DCA-2,2,4,4-D4, UDCA-2,2,4,4-D4 and CA-2,2,4,4-D4) was added in each well. The plate was vortex mixed at 1500 rpm at 10°C for 30 min and centrifuged at 4°C at 3000g for 20 min. 200 µL supernatant was vacuum-evaporated at 30°C. The residue was reconstituted with 50 µL acetonitrile and 50 µL water by vortex-mixing at 900 rpm for 20 min. After centrifugation, the plate was placed into autosampler for subsequent analysis.

In Vitro Metabolism Assay

In vitro metabolism was performed using the protocol described in our recent report (Chen et al., 2019; Zhang et al., 2019). In brief, stock solutions of BAs were prepared in DMSO. Incubations with initial substrate concentrations of 1.0, 5.0, 25, 50, 100, 200 and 300 µM were performed for DCA in 96-well plates at 37°C to acquire the oxidation kinetic data. Incubations of CA, taurocholate (TCA) and glycocholate (GCA) were comparatively performed at an initial substrate concentration of 50 µM. The 100 µL incubation solution contained 0.1 M PBS (pH 7.4), 5.0 µL NADPH-A, 1.0 µL NADPH-B, 0.5 µL BA working solution, 0.5 µL blank solvent, and 2.5 µL liver microsomes (protein concentration of 20 mg/mL). The final protein concentration in the incubation media was 0.5 mg/mL. All incubations were performed in triplicate and the DMSO concentration in all incubations was 1% (v/v). The reactions were initiated after 5 min preincubation at 37°C by adding liver microsomes and stopped at 60 min after incubation by adding 300 µL of ice-cold acetonitrile containing 0.1% formic acid and 50 µM CA-2,2,4,4-D4. The reaction mixture was then centrifuged at 4°C at 4000g for 20 minutes. For incubations using unconjugated BA as substrates, the supernatant (50 µL) was diluted with an equal volume of water and subjected to LC-MS/MS analysis. For incubations using TCA or GCA as substrates, the supernatant was vacuum-evaporated and subsequently prepared according to the enzyme digestion technique described above to hydrolyse the metabolites into unconjugated forms.

Quantitative LC-MS/MS Analysis

Quantitative analysis of unconjugated BAs was performed using previously described method with the slight modified gradient on ACQUITY ultra-performance liquid chromatography coupled to a Xevo TQ-S mass spectrometer (Waters, Milford, MA, USA) (Zhu et al., 2018). The mobile phases consisted of 0.01% formic acid in water (mobile phase A) and acetonitrile. 5 μL of each sample was injected onto an ACQUITY BEH C18 column (1.7 μm, 100 mm × 2.1 mm) (Waters, Milford, MA). The flow was 0.45 mL/min with a 16-min gradient: 0.0-0.5 min (95% A), 0.5-1.0 min (95-60% A), 1.0-2.0 min (60-74% A), 2.0-4.0 min (74-70% A), 4.0-6.0 min (70% A), 6.0-7.0 min (70-62% A), 7.0-11.0 min (62-60% A), 11.0-13.5 min (60-20% A), 13.5-14.0 min (20-0% A), 14.0-15.0 min (0% A) and 15.0-16.0 min (0-95% A). The mass spectrometer was operated in the negative mode with a 3.0 kV capillary voltage. The source and desolvation temperatures were set at 150 and 550°C, respectively. Nitrogen and argon were used as cone and collision gases, respectively. The cone gas flow and desolvation gas flow were set at 150 and 950 L/h, respectively. Selected ion recording for quantification and identification of unconjugated BAs was described in our previous reports (Yin et al., 2017; Zhu et al., 2018).

Data processing

The LC-MS/MS raw data were processed by UNIFI (V1.8, Waters, Milford, MA, USA). The concentration of urinary BA levels in individuals was corrected by creatinine (Cr) levels and expressed as nmol/mmol Cr. Multivariate analysis of the creatinine-adjusted urinary BA profiles was carried out by using SIMCA P+ (v13, Umetrics, Umeå, Sweden). Unpaired *t* tests of the creatinine-adjusted urinary levels were performed between populations by using SPSS 21.0 (IBM-SPSS Inc, IL, USA). The *in vitro* metabolite formations as a function of substrate concentrations were fit to Hill equation using GraphPad Prism software (version 7.0, GraphPad Software, LaJolla, CA).

Results

Newborns Have Urinary BA Profiles Markedly Different from Adults

A total of 40 C24 BAs were detected in urine samples of the tested populations by using the enzyme-digestion based LC-MS method. The representative ion chromatograms of the digested urine samples were shown in supplementary **Figure S1**. Conjugation patterns of the detected BAs were not investigated in this work. Among the detected BAs, thirty-one species were quantified with the in-lab authentic standards and nine unidentified tetrahydroxy-cholan-24-oic acids (Tetra-BA-1~9) were semi-quantitively determined with the calibration curve of CA. As shown in **Figure 1**, the downstream metabolism of CDCA produced a total

of thirteen known metabolites and the downstream metabolism of CA produced a total of eighteen known metabolites. **Figure 2a** showed the PCA scores plot of the creatinine-adjusted BA profiles. The PCA analysis found a poor separation between healthy adults and the pregnant, however, both of which demonstrated a clear separation from newborns. The OPLS-DA analysis confirmed the poor separation between healthy man and women (**Figure 2b**), an acceptable separation between the pregnant and healthy adults (**Figure 2c**), and strong separations between newborns and healthy adults (**Figure 2d**) or between newborns and the pregnant (**Figure 2e**).

The creatinine-adjusted urinary levels of the representative BAs detected in the tested populations were illustrated in detail in **Figure 3**, in which they were categorized in one way into primary, secondary and tertiary BAs, and in another way into the downstream metabolite of CDCA or CA. The urinary BA profiles of newborns were different from adult populations as expectations that the primary BAs were elevated due to the decreased secondary BAs in urine. This phenomenon was more significant in the downstream metabolism of CA than that of CDCA. Typical examples may be seen along the pathway from CA to DCA and from DCA to 12-epiDCA. However, the urinary levels of tertiary BAs, including the 6α-hydroxylated metabolite of LCA (HDCA) and the hydroxylated metabolites of DCA (DCA-1β-ol, DCA-6α-ol, DCA-5β-ol, and DCA-4β-ol), were not elevated in newborns as expectations. Interestingly, most of them were significantly increased in the parturient pregnant women compared to healthy non-pregnant women. DCA-19-ol, the *in vitro* marker metabolite of fetal-specific CYP3A7, was still not appreciably detected in any populations. Moreover, newborns had significantly elevated urinary levels of the 6α-hydroxylated metabolite of CDCA (αHCA) and three oxidized/epimerized metabolites of CA (3-dehydroCA, isoCA and 12-oxoCDCA). Similarly, a series of Tetra-BAs represented by five major ones (Tetra-BA-2, Tetra-BA-5~7 and Tetra-BA-9) were found as the newborn-specific BAs. These Tetra-BAs, 3-dehydroCA and 12-oxoCDCA were also found with significantly elevated levels in parturient pregnant women compared to non-pregnant healthy women, indicating that fetal-specific BAs may have an impact on the maternal BA profiles.

Urinary BA Profiles of Newborns is Characterized by Elevated Oxidations of Primary BAs.

According to the known metabolism pathways of primary, secondary and tertiary BAs in human (**Figure 4a**), the urinary metabolite ratios were calculated for the tested populations and illustrated in **Figure 4b**. In this way, the difference in the downstream metabolism of CA was particularly highlighted. The decreased secondary BA metabolism of newborns was

clearly shown by the significantly lower ratios of DCA/CA, UCA/CA, 12-epiCA/CA, 12-oxoLCA/DCA and 12-epiDCA/DCA compared to those of adults. Despite that the urinary levels of tertiary BAs were not elevated, an enhanced tertiary BA metabolism in newborns was shown by the significantly higher ratios of DCA-6α-ol/DCA, DCA-1β-ol/DCA, DCA-5β-ol/DCA and DCA-4β-ol/DCA compared to those of adults. For the downstream metabolism of CDCA, a decreased secondary BA metabolism was also manifested by the significantly lower ratio of UDCA/CDCA compared to that of adults. However, the ratio of LCA/CDCA of newborns was higher than that of adults, which was possibly associated with the decreased ratio of HDCA/LCA in newborns. This observation indicated that the microbial 7α-dehydroxylation and hepatic oxidation of the 7α-dehydroxylated metabolite may have some differences between CDCA and CA.

The metabolite ratios illustrated in **Figure 4b** clearly showed that newborns have an increased oxidation metabolism of primary BAs compared to adult populations. The ratio of αHCA/CDCA of newborns was significantly higher than that of adults, indicating that newborns had a strong CYP3A mediated 6α-hydroxylation activity toward CDCA (Deo and Bandiera, 2008). The ratios of 3-dehydroCA/CA, isoCA/CA and 12-oxoCDCA/CA, signatures of the dehydrogenation and epimerization of CA, were also significantly higher than those of adults. Moreover, the elevated oxidation of primary BAs in newborns was the most manifested by the newborn-specific Tetra-BAs. As shown in **Figure 4a**, the metabolism pathways for Tetra-BAs were believed to be associated with oxidations of trihydroxy-cholan-24-oic acids, such as CA and αHCA. However, the ratios of them to their precursors were not calculated because it was unknown whether they were derived from CA, αHCA or the other trihydroxy-cholan-24-oic acids. According to the known ontogeny of CYP3A, we deduced that the fetal specific CYP3A7 participate in the oxidation of primary BAs, particularly in the oxidation of CA and related metabolites.

Infant Liver Microsomes Have Age-Dependent Activities toward DCA 19-Oxidation.

The postnatal change from CYP3A7 toward CYP3A4 occurs rapidly in the first month and progressively during the first year after birth (Saghir et al., 2012). **Figure 4** showed an enhanced tertiary BA metabolism of newborns, which was manifested by the significantly higher urinary ratios of DCA-6α-ol/DCA, DCA-1β-ol/DCA, DCA-5β-ol/DCA and DCA-4β-ol/DCA. This observation was consistent with that the fetal CYP3A7 is more active for these reactions than CYP3A4 predominating in adult livers (Chen et al., 2019; Zhang et al., 2019). We therefore compared the DCA oxidation kinetics acquired in infant liver microsomes from

8 donors within 1-month and 3-years old to those obtained in liver microsomes from 14 adult donors. The liver microsomes of newborns were not included due to limited commercial sources. The Hill coefficients fitted within the substrate (DCA) range of 1-300 μM were shown in supplementary **Table S5**. According to the relative activities of CYP3A7 to CYP3A4 toward DCA oxidations, C-19 > C-4β > C-6α > C-1β > C-5β > C-6β (Chen et al., 2019), **Figure 5** illustrated the age-dependent intrinsic clearance (CL$_{int}$) data acquired in all the tested liver microsomes. The CYP3A7-preferring reactions to some extent showed an age-dependent decreasing tendency, such as DCA 4β-oxidation, DCA 6α-oxidation and DCA 1β-oxidation. The clearest age-dependent decrease of activities was observed for DCA 19-oxidation, the validated *in vitro* probe reaction of CYP3A7 (Chen et al., 2019). The one-month-old infant liver microsomes with the highest CYP3A7 activity were therefore used to investigated whether the metabolic enzymes in infant liver participate in CA oxidations to produce the newborns specific Tetra-BAs.

Infant Liver Microsomes Oxidize CA More Than Adult Liver Microsomes.

In vitro oxidation metabolism of CA was investigated to clarify whether the fetal-specific Tetra-BAs are hepatic oxidized metabolites of primary BAs. Incubations of CA, TCA and GCA were performed in parallel because the taurine conjugates of BAs predominated in newborns (Poley et al., 1964). We firstly compared the oxidation activities of one-month-old infant liver microsomes (HLM-IF) and Human liver microsomes from 150 (76 female and 74 male) mixed gender pooled donors (HLM) to mimic the hepatic metabolism of newborns and adults. **Figure 6a** showed the metabolite formations after incubations of CA, TCA and GCA at 50 μM in HLM and HLM-IF. The oxidized metabolites of GCA and TCA were detected after being digested by choloylglycine hydrolase (Zhu et al., 2018). Four oxidative metabolites of CA were detected in the incubation samples, including Tetra-BA-2, Tetra-BA-3, Tetra-BA-5 and Tetra-BA-7, whose retention time matched well with those detected in the urines of newborns (supplementary **Table S6**). Anyway, these Tetra-BAs were minor metabolites compared to the dehydrogenated metabolite, 3-dehyroCA, which was the major *in vitro* metabolite of CA, TCA and GCA. The 3-dehydrogenation mechanism were considered to be involved with a 3α,3β-diol intermediate formed by 3β-oxidation according to the 3-dehydrogenation mechanism in DCA oxidation (Bodin et al., 2005; Zhang et al., 2019). The other fetal-specific Tetra-BAs, 12-oxoCDCA and isoCA were not detected in the *in vitro* incubation samples, indicated that they may not be the hepatic metabolites of CA, TCA and GCA.

As shown in **Figure 6a**, HLM-IF exhibited much higher activities than HLM toward the oxidation reactions to produce Tetra-BA-2, Tetra-BA-3, Tetra-BA-5. For these reactions, both HLM-IF and HLM showed higher oxidation activities toward TCA than CA and GCA. This data partly explained why the three Tetra-BAs were detected in newborns but not found in adults because CA is mainly conjugated with taurine in newborns. However, HLM-IF and HLM showed comparable activities toward the 3β-oxidation of TCA and GCA as well as the oxidation reactions to produce Tetra-BA-7. Therefore, the in vitro metabolism assay in liver microsomes did not explain the fetal-specific urinary excretion of 3-dehydroCA and Tetra-BA-7 compared to adults. Further studies should consider whether the intestinal epithelia or infant gut microbes contribute to the fetal-specific oxidative metabolism of primary BAs.

Limited Contributions of Recombinant CYP3A7 to Cholate Oxidations

In order to clarify whether CYP3A7 in HLM-IF contribute to the oxidation reactions to produce Tetra-BA-2, Tetra-BA-3, Tetra-BA-5, we subsequently compared the oxidation activities of recombinant CYP3A4, 3A5 and 3A7 enzymes for CA, TCA and GCA (**Figure 6b**). The recombinant P450 enzyme assay did not show consistency to the observations in HLM-IF and HLM except for the 3β-oxidation, toward which CYP3A4, 3A5 and 3A7 had comparable activities for the substrates of TCA and GCA to produce 3-dehydrogenated metabolites. Comparable activities of CYP3A isoenzymes had also been shown toward the 3β-oxidation of GDCA and TDCA to produce 3-dehydrogenated metabolites (Zhang et al., 2019). Compared to CYP3A4 and 3A7, CYP3A5 had none or trace activity for the oxidative reactions to produce Tetra-BAs. However, CYP3A7 did not exhibit stronger oxidation activities than CYP3A4 as expectations for the oxidation reactions to produce Tetra-BA-2, Tetra-BA-3, Tetra-BA-5, indicating that the fetal-specific CYP3A7 may play a limited role in the oxidation metabolism of CA, TCA and GCA in newborns.

Discussion

It has been known for a long time that the BA metabolism of infants is different from that of adults (Poley et al., 1964). Numerous literatures published decades before had found a series of fetal-specific BAs with "unusual" oxidation sites including C-1β, -2β, -4β, -6α/β and -19 in early human life. Summary of these literature may be seen in a recent book (Sjövall et al., 2010). However, it remains undefined that how and to what extent the fetal BA metabolism is different from that of adults along the axis of primary, secondary and tertiary BAs. It's accordingly uncharted what is the driving force for the transition from the neonatal BA metabolism phenotype to that of adults, what is the driving force for the ontogeny of

P450s that play important roles in both lipid metabolism and pediatric pharmacology, and what's the relationship between the development of human BA's host-gut microbial co-metabolism and metabolic diseases during pregnancy and childhood. In this regard, the revisiting studies of the perinatal BA metabolism are imperative in the era of metagenomics. Such results obtained in murine animals should be carefully extrapolated to human because they have BA metabolism pathways markedly different from human (Thakare et al., 2018b; Thakare et al., 2018a; Lin et al., 2020). In human studies, however, samples of newborns and infants are available mainly in diaper disposals due to ethic and practical issues. Meconium is hard to be precisely analysed because it rapidly losses water on diapers and contaminates the urine samples. We therefore developed and validated a protocol to isolate the diaper urine samples without meconium contamination. The $CaCl_2$ method showed high efficiency and acceptable accuracy to extract urinary BAs absorbed in the diaper gel. Using this method, we were able to compare the urinary BA profiles of newborns to those of adult controls. The newborns of cesarean section were enrolled in this work because diapers are hard to be collected from naturally labored babies, who usually left the hospital 24 hours after delivery.

By using BA profiling technique in assistance with the *in vitro* metabolism assays, this work disclosed that the urinary BA profiles of newborns are characterized by the elevated oxidative metabolism of primary BA compared to those of adults. Deeper understanding of the difference in BA conjugation metabolism relies on development of the next-generation BA profiling techniques including not only N-amidated forms but also sulfated and glucuronidated forms of BAs. This metabolic phenotype seemed to be more significant in the downstream of CA, which was manifested by the significantly elevated urinary ratios of 3-dehydroCA/CA, isoCA/CA and 12-oxoCDCA/CA, than that of CDCA, which was indicated by the significantly increased urinary ratios of αHCA/CDCA. High levels of 3-dehydroCA have been previously reported in amniotic fluid of pregnant women (Nakagawa and Setchell, 1990), urine of newborns (Kimura et al., 1997), preterm and full-term infants (Maeda et al., 2003). Moreover, such phenotype was more clearly shown by the nine fetal-specific Tetra-BAs, which have been mentioned decades before in urine of patients with hepatobiliary diseases (Alme et al., 1977) and newborns (Strandvik and Wikstrom, 1982). Among these Tetra-BAs, four metabolites (Tetra-BA-2, Tetra-BA-3, Tetra-BA-5 and Tetra-BA-7) were oxidized metabolites of CA in infant liver microsomes. Metabolism pathways for the other Tetra-BAs were believed to be associated with hepatic or extrahepatic oxidations of the major trihydroxy-cholan-24-oic acids of newborns. Chemical structures of these Tetra-BAs were not identified in this work due to time-consuming synthesis of the potential authentic

standards. According to the related literatures (Tohma et al., 1985; Kimura et al., 1994), the major Tetra-BAs were believed to be associated with the 1β-hydroxylated metabolites of CA or the subsequent hydroxylated metabolites of the oxidized metabolites of CDCA, such as αHCA and CDCA-1β-ol. The other Tetra-BAs were proposed to be metabolites with oxidation sites on A or B ring (Dumaswala et al., 1989; Kurosawa et al., 1989), which is similar to those of DCA disclosed in our publications (Chen et al., 2019; Zhang et al., 2019).

The in-depth analysis of urinary BA profiles also found a decreased secondary BAs metabolism in newborns coincided with their elevated primary BAs oxidation because they have limited colonization of gut bacteria responsible for secondary BA production. Accordingly, the tertiary BA metabolism, which may play a complementary role in the disposition of secondary BA in adults, also did not contribute significantly to the renal excretion of BAs in newborns, despite that the newborns exhibited enhanced tertiary BA metabolism, which was indicated by the elevated ratios of DCA-6α-ol/DCA, DCA-1β-ol/DCA, DCA-5β-ol/DCA and DCA-4β-ol/DCA. The phenotype of increased tertiary BA metabolism is consistent with that the newborns have much higher hepatic activities of fetal CYP3A7, which has stronger activity toward DCA oxidation than CYP3A4 that dominates in adult livers. DCA-19-ol, the major oxidized metabolite of DCA in infant microsomes (**Figure 5**), was also not appreciably detected in urine samples of newborns as expectations. The *in vitro - in vivo* inconsistency of DCA 19-hydroxylation reinforced our previous deduction about the subsequent conversion of DCA-19-ol into another unknown metabolite (Chen et al., 2019; Zhang et al., 2019). It has to be mentioned once again that the newborns of cesarean section rather than natural birth were enrolled in this work. It's known that the delivery method play a decisive role in the first colonization of gut bacteria in newborns and the subsequent developments of gut microbiota in infants (Chu et al., 2017). Such a disparity in delivery mode may result in different secondary BA metabolism and the subsequent tertiary BA metabolism. Future works are needed to decipher the phenotypic difference of BA metabolism between cesarean section newborns and natural birth newborns.

According to the BA metabolism phenotype of cesarean sectioned newborns, we believed that the hepatic oxidations of primary BAs might have played a vital role in BA elimination in early life. This deduction was also evident by the fact that these fetal-specific BAs were significantly elevated in urine of parturient pregnant women compared to non-pregnant healthy women. The examples included all the Tetra-BAs, DCA-6α-ol (tertiary BA), DCA-1β-ol (tertiary BA), DCA-4β-ol (tertiary BA), 3-dehydroCA (fetal-specific BA), 12-oxoCDCA (fetal-specific BA) in the downstream metabolism of CA, as well as HDCA

(tertiary BA) in the downstream metabolism of CDCA. The significantly elevated urinary excretion of these BAs in parturient pregnant women indicated that oxidized metabolites of primary BAs and the tertiary BA metabolites produced by fetus may be excreted into amniotic fluid through meconium and fetal urine for maternal disposition. Simultaneous analysis of the BA profiles in maternal amniotic fluid, urine and feces in comparison to meconium and urine of newborns and infants may provide fundamental information about whether such a maternal-fetal interplay of BA disposition is associated with perinatal cholestasis associated diseases.

This work performed preliminary *in vitro* metabolism studies to investigate whether the fetal specific CYP3A7 contributes to the elevated oxidative metabolism of primary BA in newborns, exemplified by the substrates of CA, TCA and GCA. The HLM-IF from a one-month-old donor, which was characterized by DCA 19-oxidation to have the highest CYP3A7 activities (Chen et al., 2019), was employed as alternative of newborn liver fractions. HLM-IF exhibited much higher activities than HLM of pooled adult donors, particularly toward the oxidation reactions of TCA to produce Tetra-BA-2, Tetra-BA-3, Tetra-BA-5. It's unclear to what extent the difference occurs in human intestine microsomes due to no commercial source of infant intestine microsomes. Beyond our expectation, however, the recombinant enzyme assays did not show that CYP3A7 significantly contributed to these reactions compared to CYP3A4. CYP3A5 showed no or trace activities to these reactions except for the 3-dehydrogenation reaction of TCA and GCA. We therefore concluded that the fetal-specific CYP3A7 might have played a limited role in the oxidation metabolism of CA, TCA and GCA in newborns. Since most drug-metabolizing P450 isoforms, including CYP1A2, CYP2A6, CYP2B6, CYP2D6, CYP2C9, CYP2C19, CYP1E2 and CYP3A4, express limitedly in newborn livers and have the similar postnatal ontogenic pattern (Hines, 2007; Upreti and Wahlstrom, 2016; Allegaert and van den Anker, 2019), it is deduced that some other fetal-specific P450 isoforms may participate the oxidations of CA, GCA and particularly TCA.

In conclusion, this work demonstrated how the BA metabolism of cesarean sectioned newborns differs from that of adults along the axis of primary, secondary and tertiary BAs. Due to lack of developed gut microbiota, newborns have poor secondary BAs metabolism that plays a vital role in BAs disposition of adults. Subsequently, the tertiary metabolism of secondary BAs contributes limitedly to the disposition of BAs in newborns. As a result, the primary BAs synthesized in the infant liver undergo extensive oxidation metabolism, resulting in significantly elevated levels of the oxidized metabolites in newborn urine, such as

αHCA, 3-dehydroCA, isoCA and 12-oxoCDCA and several Tetra-BAs, some of which were characterized as hydroxylated metabolites of CA by *in vitro* metabolism assay in infant liver microsomes. Compared to CYP3A4, the fetal specific CYP3A7 did not contribute to CA oxidation as much as expectation. It is therefore deduced that the fetal-specific BA disposition pattern is gradually translated into the metabolism axis of primary, secondary and tertiary BAs of adults with the developments of gut microbiota and the ontogeny of P450 enzymes after birth. Comprehensive technologies involving metagenomics, transcriptomics, proteomics, metabolomics and *in vitro* activity assays may be helpful to decipher the mechanism underlying the fetal specific BA metabolism phenotype and its transition to the adult phenotype. Such knowledge is of particular importance in both pediatric pharmacology and molecular pathology of perinatal diseases, such as perinatal cholestasis diseases, and is promising to be linked to other ontogeny and development since BAs play important roles in lipid absorption and regulation of lipid/carbohydrate metabolism.

Acknowledgments

Authorship Contributions

Participated in research design: Lan K.

Conducted experiments: Wang WX, Chen L, Wang GY, Zhang LJ, Tan XW, Lin QH, Chen YJ, Zhang J, Zhu PP, Miao J, Su MM.

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Wrote or contributed to the writing of the manuscript: Wang WX, Chen L, Liu CX, Jia W, Lan K.

References

Allegaert K and van den Anker J (2019) Ontogeny of Phase I Metabolism of Drugs. *J Clin Pharmacol* **59 Suppl 1:**S33-S41.

Alme B, Bremmelgaard A, Sjovall J, and Thomassen P (1977) Analysis of metabolic profiles of bile acids in urine using a lipophilic anion exchanger and computerized gas-liquid chromatorgaphy-mass spectrometry. *J Lipid Res* **18:**339-362.

Alme B and Sjovall J (1980) Analysis of bile acid glucuronides in urine. Identification of 3 alpha, 6 alpha, 12 alpha-trihydroxy-5 beta-cholanoic acid. *J Steroid Biochem* **13:**907-916.

Batta AK, Arora R, Salen G, Tint GS, Eskreis D, and Katz S (1989) Characterization of serum and urinary bile acids in patients with primary biliary cirrhosis by gas-liquid chromatography-mass spectrometry: effect of ursodeoxycholic acid treatment. *J Lipid Res* **30:**1953-1962.

Bodin K, Lindbom U, and Diczfalusy U (2005) Novel pathways of bile acid metabolism involving CYP3A4. *Biochim Biophys Acta* **1687:**84-93.

Chen YJ, Zhang J, Zhu PP, Tan XW, Lin QH, Wang WX, Yin SS, Gao LZ, Su MM, Liu CX, Xu L, Jia W, Sevrioukova IF, and Lan K (2019) Stereoselective Oxidation Kinetics of Deoxycholate in Recombinant and Microsomal CYP3A Enzymes: Deoxycholate 19-Hydroxylation Is an In Vitro Marker of CYP3A7 Activity. *Drug Metab Dispos* **47:**574-581.

Chu DM, Ma J, Prince AL, Antony KM, Seferovic MD, and Aagaard KM (2017) Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery. *Nat Med* **23:**314-326.

Dawson PA and Karpen SJ (2015) Intestinal transport and metabolism of bile acids. *J Lipid Res* **56:**1085-1099.

Deo AK and Bandiera SM (2008) Identification of human hepatic cytochrome p450 enzymes involved in the biotransformation of cholic and chenodeoxycholic acid. *Drug Metab Dispos* **36:**1983-1991.

Dumaswala R, Setchell KD, Zimmer-Nechemias L, Iida T, Goto J, and Nambara T (1989) Identification of 3 alpha,4 beta,7 alpha-trihydroxy-5 beta-cholanoic acid in human bile: reflection of a new pathway in bile acid metabolism in humans. *J Lipid Res* **30:**847-856.

Halilbasic E, Claudel T, and Trauner M (2013) Bile acid transporters and regulatory nuclear receptors in the liver and beyond. *J Hepatol* **58:**155-168.

Hines RN (2007) Ontogeny of human hepatic cytochromes P450. *J Biochem Mol Toxicol* **21:**169-175.

Hu Y, Beach J, Raymer J, and Gardner M (2004) Disposable diaper to collect urine samples from young children for pyrethroid pesticide studies. *J Biochem Mol Toxicol* **14:**378-384.

Ikegawa S, Murai T, Matsui A, and Tohma M (1994) Radioimmunoassay of conjugated 1 beta-hydroxycholic acid in dried blood spots for diagnosis of congenital biliary atresia. *Biol Pharm Bull* **17:**5-8.

Kakiyama G, Muto A, Takei H, Nittono H, Murai T, Kurosawa T, Hofmann AF, Pandak WM, and Bajaj JS (2014) A simple and accurate HPLC method for fecal bile acid profile in healthy and cirrhotic subjects: validation by GC-MS and LC-MS. *J Lipid Res* **55:**978-990.

Kimura A, Suzuki M, Murai T, Inoue T, Kato H, Hori D, Nomura Y, Yoshimura T, Kurosawa T, and Tohma M (1997) Perinatal bile acid metabolism: analysis of urinary bile acids in pregnant women and newborns. *J Lipid Res* **38:**1954-1962.

Kimura A, Yamakawa R, Ushijima K, Fujisawa T, Kuriya N, Kato H, Inokuchi T, Mahara R, Kurosawa T, and Tohma M (1994) Fetal bile acid metabolism during infancy: analysis of 1 beta-hydroxylated bile acids in urine, meconium and feces. *Hepatology* **20:**819-824.

Kivisto KT, Bookjans G, Fromm MF, Griese EU, Munzel P, and Kroemer HK (1996) Expression of CYP3A4, CYP3A5 and CYP3A7 in human duodenal tissue. *Br J Clin Pharmacol* **42:**387-389.

Kumagai M, Kimura A, Takei H, Kurosawa T, Aoki K, Inokuchi T, and Matsuishi T (2007) Perinatal bile acid metabolism: bile acid analysis of meconium of preterm and full-term infants. *J Gastroenterol* **42:**904-910.

Kurosawa T, Mahara R, Nittono H, and Tohma M (1989) Synthesis of 6-hydroxylated bile acids and identification of 3 alpha,6 alpha,7 alpha,12 alpha-tetrahydroxy-5 beta-cholan-24-oic acid in human meconium and neonatal urine. *Chem Pharm Bull (Tokyo)* **37:**557-559.

Kurosawa T, Nomura Y, Mahara R, Yoshimura T, Kimura A, Ikegawa S, and Tohma M (1995) Synthesis of 19-hydroxylated bile acids and identification of 3 alpha,7 alpha,12 alpha,19-tetrahydroxy-5 beta-cholan-24-oic acid in human neonatal urine. *Chem Pharm Bull (Tokyo)* **43:**1551-1557.

Lin Q, Tan X, Wang W, Zeng W, Gui L, Su M, Liu C, Jia W, Xu L, and Lan K (2020) Species differences of bile acid redox metabolism: tertiary oxidation of deoxycholate is conserved in preclinical animals. *Drug Metab Dispos* **accepted**.

Liu L, Xia T, Zhang X, Barr DB, Alamdar A, Zhang J, Tian M, Huang Q, and Shen H (2014) Biomonitoring of infant exposure to phenolic endocrine disruptors using urine expressed from disposable gel diapers. *Anal Bioanal Chem* **406:**5049-5054.

Maeda K, Kimura A, Yamato Y, and Matsuishi T (2003) Perinatal bile acid metabolism: analysis of urinary unsaturated ketonic bile acids in preterm and full-term infants. *Acta Paediatr* **92:**216-220.

Mahara R, Takeshita H, Kurosawa T, Ikegawa S, and Tohma M (1987) Determination of 1.BETA.-hydroxylated bile acids and related compounds in human biological fluids by gas chromatography-mass spectrometry. *Anal Sci* **3**: 449-452.

Meng LJ, Reyes H, Palma J, Hernandez I, Ribalta J, and Sjovall J (1997a) Effects of ursodeoxycholic acid on conjugated bile acids and progesterone metabolites in serum and urine of patients with intrahepatic cholestasis of pregnancy. *J Hepatol* **27:**1029-1040.

Meng LJ, Reyes H, Palma J, Hernandez I, Ribalta J, and Sjovall J (1997b) Profiles of bile acids and progesterone metabolites in the urine and serum of women with intrahepatic cholestasis of pregnancy. *J Hepatol* **27:**346-357.

Miyara T, Shindo N, Tohma M, and Murayama K (1990) Capillary gas chromatography/negative ion chemical ionization mass spectrometry for the quantification of bile acids including 1 beta-hydroxylated and unsaturated bile acids in serum and urine. *Biomed Chromatogr* **4:**56-60.

Nakagawa M and Setchell KD (1990) Bile acid metabolism in early life: studies of amniotic fluid. *J Lipid Res* **31:**1089-1098.

Nishiura H, Kimura A, Yamato Y, Aoki K, Inokuchi T, Kurosawa T, and Matsuishi T (2010) Developmental pattern of urinary bile acid profile in preterm infants. *Pediatr Int* **52:**44-50.

Poley JR, Dower JC, Owen CA, Jr., and Stickler GB (1964) Bile Acids in Infants and Children. *J Lab Clin Med* **63:**838-846.

Russell DW (2003) The enzymes, regulation, and genetics of bile acid synthesis. *Annu Rev Biochem* **72:**137-174.

Saghir SA, Khan SA, and McCoy AT (2012) Ontogeny of mammalian metabolizing enzymes in humans and animals used in toxicological studies. *Crit Rev Toxicol* **42:**323-357.

Schuetz JD, Kauma S, and Guzelian PS (1993) Identification of the fetal liver cytochrome CYP3A7 in human endometrium and placenta. *J Clin Invest* **92:**1018-1024.

Setchell KD, Dumaswala R, Colombo C, and Ronchi M (1988) Hepatic bile acid metabolism during early development revealed from the analysis of human fetal gallbladder bile. *J Biol Chem* **263:**16637-16644.

Shoda J, Mahara R, Osuga T, Tohma M, Ohnishi S, Miyazaki H, Tanaka N, and Matsuzaki Y (1988) Similarity of unusual bile acids in human umbilical cord blood and amniotic fluid from newborns and in sera and urine from adult patients with cholestatic liver diseases. *J Lipid Res* **29:**847-858.

Shoda J, Osuga T, Mahara R, Tohma M, Matsuura K, Tanaka N, Matsuzaki Y, and Miyazaki H (1989) Altered metabolism of bile acids in cholestasis: determination of 1 beta- and 6 alpha-hydroxylated metabolites. *J Chromatogr* **488:**315-328.

Shoda J, Tanaka N, Osuga T, Matsuura K, and Miyazaki H (1990) Altered bile acid metabolism in liver disease: concurrent occurrence of C-1 and C-6 hydroxylated bile acid metabolites and their preferential excretion into urine. *J Lipid Res* **31:**249-259.

Sjövall J, Griffiths WJ, Setchell KDR, Mano N, and Goto J (2010) *Analysis of Bile Acids*. Springer.

Strandvik B and Wikstrom SA (1982) Tetrahydroxylated bile acids in healthy human newborns. *Eur J Clin Invest* **12:**301-305.

Suzuki M, Murai T, Yoshimura T, Kimura A, Kurosawa T, and Tohma M (1997) Determination of 3-oxo-delta4- and 3-oxo-delta4,6-bile acids and related compounds in biological fluids of infants with cholestasis by gas chromatography-mass spectrometry. *J Chromatogr B* **693:**11-21.

Thakare R, Alamoudi JA, Gautam N, Rodrigues AD, and Alnouti Y (2018a) Species differences in bile acids I. Plasma and urine bile acid composition. *J Appl Toxicol* **38:**1323-1335.

Thakare R, Alamoudi JA, Gautam N, Rodrigues AD, and Alnouti Y (2018b) Species differences in bile acids II. Bile acid metabolism. *J Appl Toxicol* **38:**1336-1352.

Tohma M, Mahara R, Takeshita H, Kurosawa T, and Ikegawa S (1986) Synthesis of the 1 beta-hydroxylated bile acids, unusual bile acids in human biological fluids. *Chem Pharm Bull (Tokyo)* **34:**2890-2899.

Tohma M, Mahara R, Takeshita H, Kurosawa T, Ikegawa S, and Nittono H (1985) Synthesis of the 1 beta-hydroxylated bile acids and identification of 1 beta,3 alpha,7 alpha-trihydroxy- and 1 beta,3 alpha,7 alpha,12 alpha-tetrahydroxy-5 beta-cholan-24-oic acids in human meconium. *Chem Pharm Bull (Tokyo)* **33:**3071-3073.

Upreti VV and Wahlstrom JL (2016) Meta-analysis of hepatic cytochrome P450 ontogeny to underwrite the prediction of pediatric pharmacokinetics using physiologically based pharmacokinetic modeling. *J Clin Pharmacol* **56:**266-283.

Yin S, Su M, Xie G, Li X, Wei R, Liu C, Lan K, and Jia W (2017) Factors affecting separation and detection of bile acids by liquid chromatography coupled with mass spectrometry in negative mode. *Anal Bioanal Chem* **409:**5533-5545.

Zhang J, Gao LZ, Chen YJ, Zhu PP, Yin SS, Su MM, Ni Y, Miao J, Wu WL, Chen H, Brouwer KLR, Liu CX, Xu L, Jia W, and Lan K (2019) Continuum of Host-Gut Microbial Co-metabolism: Host CYP3A4/3A7 Are Responsible for Tertiary Oxidations of Deoxycholate Species. *Drug Metab Dispos* **47:**283-294.

Zhu P, Zhang J, Chen Y, Yin S, Su M, Xie G, Brouwer KLR, Liu C, Lan K, and Jia W (2018) Analysis of human C24 bile acids metabolome in serum and urine based on enzyme digestion of conjugated bile acids and LC-MS determination of unconjugated bile acids. *Anal Bioanal Chem* **410:**5287-5300.

Footnotes

Figure Legends

Figure 1. Chemical structures of forty unconjugated BAs and their creatinine adjusted urinary levels in healthy man (n=30), non-pregnant healthy women (n=28), parturient pregnant women (n=29) and cesarean newborns (n=21). Data were shown as mean±SEM.

Figure 2. Multivariate analysis of the creatinine-adjusted urinary BA profiles of healthy man (n=30), non-pregnant healthy women (n=28), parturient pregnant women (n=29) and cesarean sectioned newborns (n=21). PCA scores plot of all population (a), OPLS-DA scores plot between man and women (b), OPLS-DA scores plot between healthy adults and the pregnant (c), OPLS-DA scores plot between healthy adults and newborns (d), OPLS-DA scores plot between the pregnant and newborns (e).

Figure 3. The creatinine-adjusted urinary levels of representative unconjugated BAs in healthy man (n=30), non-pregnant healthy women (n=28), parturient pregnant women (n=29) and cesarean sectioned newborns (n=21). Data were shown as median with 95% confidential interval. Unpaired t tests were performed between populations (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

Figure 4. Metabolic pathways of bile acids in human (a) and their urinary ratios in healthy man (n=30), non-pregnant healthy women (n=28), parturient pregnant women (n=29) and cesarean newborns (n=21). Data were shown as median with 95% confidential interval. Unpaired t tests were performed between populations (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

Figure 5. The *in vitro* intrinsic clearance (CL_{int}) of DCA oxidations in the infant liver microsomes from eight single donors aged from 1-month to 3-years old and the adult liver microsomes from fourteen single donors. Data were shown as mean±SD for adult liver microsomes.

Figure 6. *In vitro* metabolite formation after incubation of cholate (CA), taurocholate (TCA) and glycocholate (GCA) at 50 μM in liver microsomes (a) and recombinant CYP3A enzymes (b) for 60 min. Data were shown as mean±SD (n=3) in pmol/min/mg protein for adult liver microsomes (HLM) and infant liver microsomes (HLM-IF), which were incubated at a protein level of 0.5 mg/mL, and in pmol/min/nmol P450 for the recombinant CYP3A4, 3A5 and 3A7 enzymes incubated at a protein level of 50 pmol protein/mL.

	Name	R₁	R₃	R₄	R₅	R₆	R₇	R₁₂	R₁₉	R₂₄
	CDCA	H	α-OH	H	H	H	α-OH	H	H	OH
	αHCA	H	α-OH	H	H	α-OH	α-OH	H	H	OH
	αMCA	H	α-OH	H	H	β-OH	α-OH	H	H	OH
	7-oxoLCA	H	α-OH	H	H	H	=O	H	H	OH
CDCA metabolites	UDCA	H	α-OH	H	H	H	β-OH	H	H	OH
	isoUDCA	H	β-OH	H	H	H	β-OH	H	H	OH
	βHCA	H	α-OH	H	H	α-OH	β-OH	H	H	OH
	βMCA	H	α-OH	H	H	β-OH	β-OH	H	H	OH
	LCA	H	α-OH	H	H	H	H	H	H	OH
	MDCA	H	α-OH	H	H	β-OH	H	H	H	OH
	6-oxoLCA	H	α-OH	H	H	=O	H	H	H	OH
	HDCA	H	α-OH	H	H	α-OH	H	H	H	OH
	isoHDCA	H	β-OH	H	H	α-OH	H	H	H	OH
	CA	H	α-OH	H	H	H	α-OH	α-OH	H	OH
	3-dehydroCA	H	=O	H	H	H	α-OH	α-OH	H	OH
	isoCA	H	β-OH	H	H	H	α-OH	α-OH	H	OH
	7-oxoDCA	H	α-OH	H	H	H	=O	α-OH	H	OH
	UCA	H	α-OH	H	H	H	β-OH	α-OH	H	OH
	isoUCA	H	β-OH	H	H	H	β-OH	α-OH	H	OH
	12-oxoCDCA	H	α-OH	H	H	H	α-OH	=O	H	OH
	12-epiCA	H	α-OH	H	H	H	α-OH	β-OH	H	OH
CA metabolites	DCA	H	α-OH	H	H	H	H	α-OH	H	OH
	isoDCA	H	β-OH	H	H	H	H	α-OH	H	OH
	12-oxoLCA	H	α-OH	H	H	H	H	=O	H	OH
	12-epiDCA	H	α-OH	H	H	H	H	β-OH	H	OH
	DCA-19-ol	H	α-OH	H	H	H	H	α-OH	OH	OH
	DCA-6β-ol	H	α-OH	H	H	β-OH	H	α-OH	H	OH
	DCA-5β-ol	H	α-OH	H	OH	H	H	α-OH	H	OH
	DCA-6α-ol	H	α-OH	H	H	α-OH	H	α-OH	H	OH
	DCA-1β-ol	β-OH	α-OH	H	H	H	H	α-OH	H	OH
	DCA-4β-ol	H	α-OH	β-OH	H	H	H	α-OH	H	OH

Figure 1

Figure 2

Figure 3
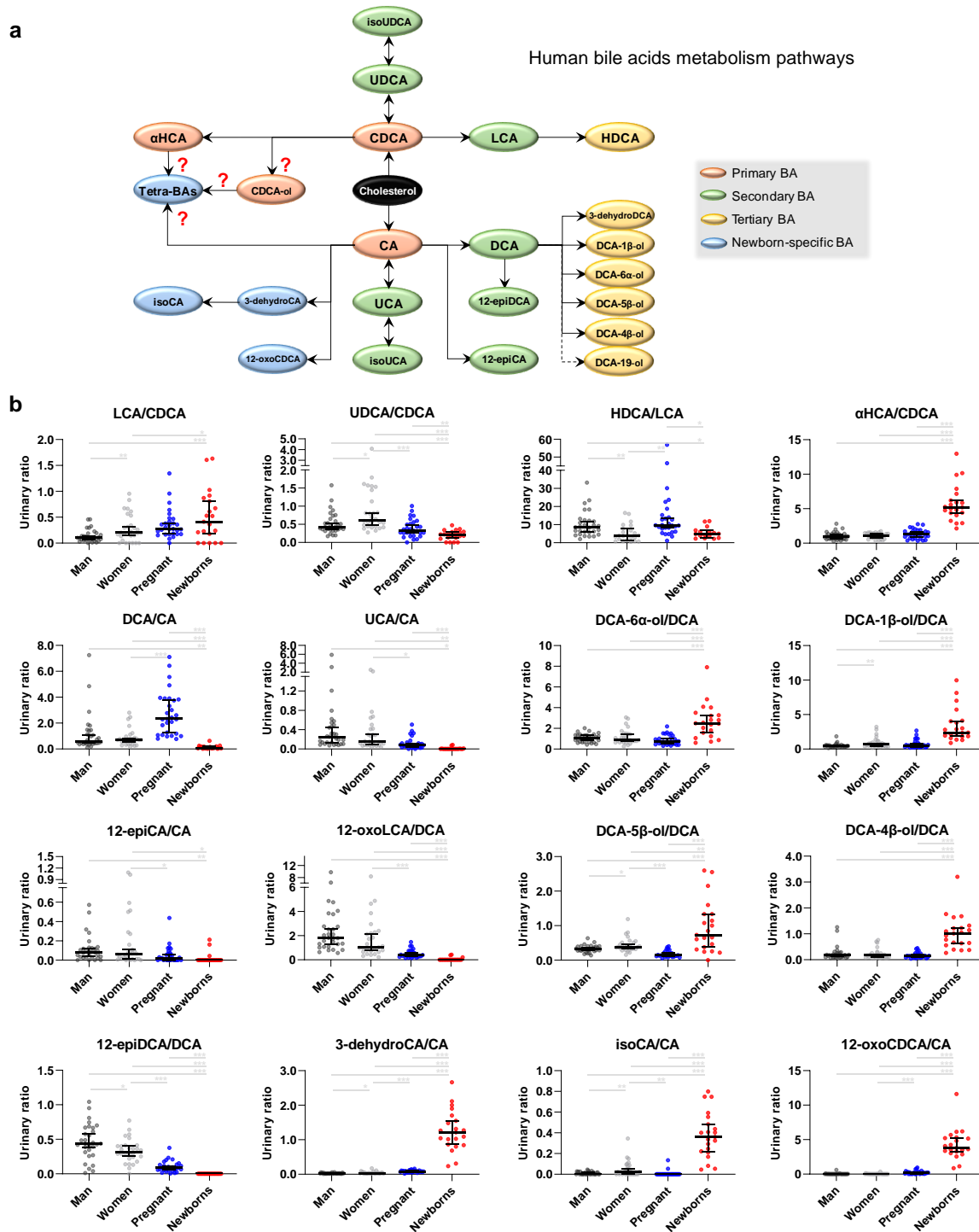
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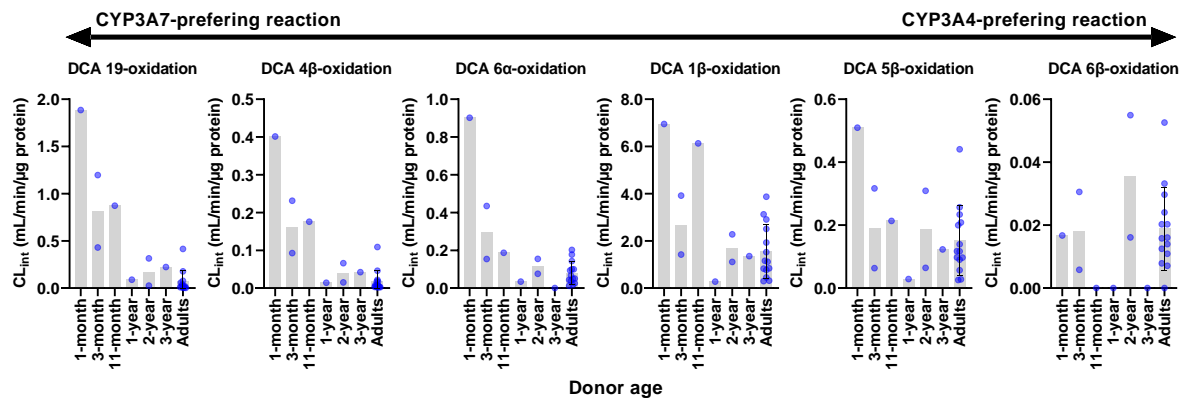
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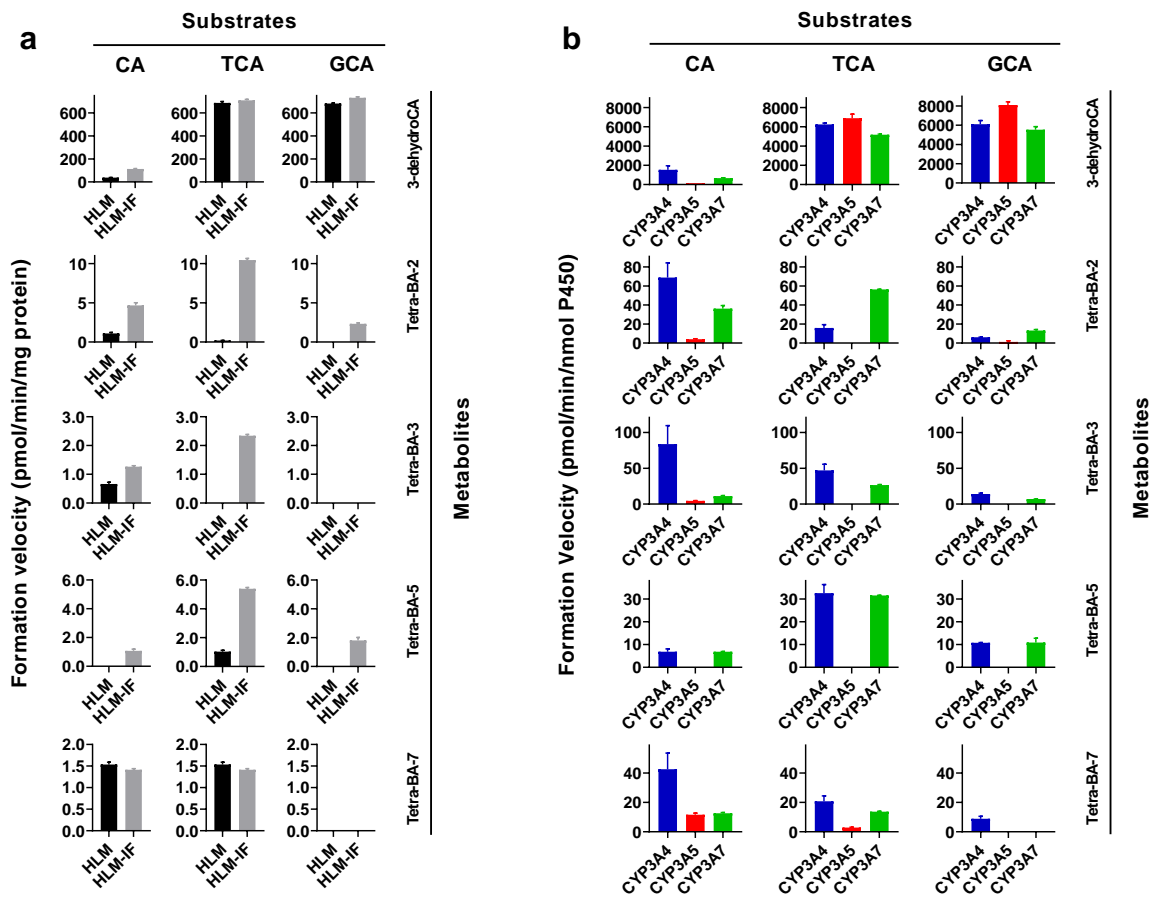
Figure 6
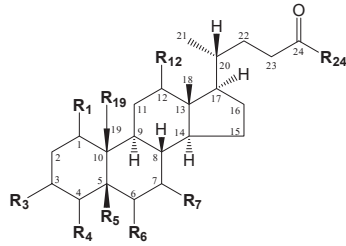
Name	R_1	R_3	R_4	R_5	R_6	R_7	R_{12}	R_{19}	R_{24}
CDCA metabolites									
CDCA	H	α-OH	H	H	H	α-OH	H	H	OH
αHCA	H	α-OH	H		α-OH	α-OH	H	H	OH
αMCA	H	α-OH	H		β-OH	α-OH	H	H	OH
7-oxoLCA	H	α-OH	H	H	H	=O	H	H	OH
UDCA	H	α-OH	H	H	H	β-OH	H	H	OH
isoUDCA	H	β-OH	H		H	β-OH	H	H	OH
βHCA	H	α-OH	H		α-OH	β-OH	H	H	OH
βMCA	H	α-OH	H		β-OH	β-OH	H	H	OH
LCA	H	α-OH	H	H	H	H	H	H	OH
MDCA	H	α-OH	H		β-OH	H	H	H	OH
6-oxoLCA	H	α-OH	H	H	=O	H	H	H	OH
HDCA	H	α-OH	H		α-OH	H	H	H	OH
isoHDCA	H	β-OH	H		α-OH	H	H	H	OH
CA metabolites									
CA	H	α-OH	H	H	H	α-OH	α-OH	H	OH
3-dehydroCA	H	=O	H	H	H	α-OH	α-OH	H	OH
isoCA	H	β-OH	H	H	H	α-OH	α-OH	H	OH
7-oxoDCA	H	α-OH	H	H	H	=O	α-OH	H	OH
UCA	H	α-OH	H	H	H	β-OH	α-OH	H	OH
isoUCA	H	β-OH	H	H	H	β-OH	α-OH	H	OH
12-oxoCDCA	H	α-OH	H	H	H	α-OH	=O	H	OH
12-epiCA	H	α-OH	H	H	H	α-OH	β-OH	H	OH
DCA	H	α-OH	H	H	H	H	α-OH	H	OH
isoDCA	H	β-OH	H	H	H	H	α-OH	H	OH
12-oxoLCA	H	α-OH	H	H	H	H	=O	H	OH
12-epiDCA	H	α-OH	H	H	H	H	β-OH	H	OH
DCA-19-ol	H	α-OH	H	H	H	H	α-OH	OH	OH
DCA-6β-ol	H	α-OH	H		β-OH	H	α-OH	H	OH
DCA-5β-ol	H	α-OH	H	OH	H	H	α-OH	H	OH
DCA-6α-ol	H	α-OH	H		α-OH	H	α-OH	H	OH
DCA-1β-ol	β-OH	α-OH	H	H	H	H	α-OH	H	OH
DCA-4β-ol	H	α-OH	β-OH	H	H	H	α-OH	H	OH

Legend: Primary BA · Secondary BA · Tertiary BA · Newborns-specific BA

Man (n=30) — nmole/mmole Cr.
CDCA metabolites | CA metabolites | Tetra-OH BAs

Woman (n=28) — nmole/mmole Cr.
CDCA metabolites | CA metabolites | Tetra-OH BAs

Pregnant (n=29) — nmole/mmole Cr.
CDCA metabolites | CA metabolites | Tetra-OH BAs

Newborn (n=21) — nmole/mmole Cr.
CDCA metabolites | CA metabolites | Tetra-OH BAs

X-axis categories: CDCA, αHCA, αMCA, 7-oxoLCA, UDCA, isoUDCA, βHCA, βMCA, LCA, MDCA, 6-oxoLCA, HDCA, isoHDCA, CA, 3-dehydroCA, isoCA, 7-oxoDCA, UCA, isoUCA, 12-oxoCDCA, 12-epiCA, DCA, isoDCA, 12-oxoLCA, 12-epiDCA, DCA-19-ol, DCA-6β-ol, DCA-5β-ol, DCA-6α-ol, DCA-1β-ol, DCA-4β-ol, Tetra-BA-1, Tetra-BA-2, Tetra-BA-3, Tetra-BA-4, Tetra-BA-5, Tetra-BA-6, Tetra-BA-7, Tetra-BA-8, Tetra-BA-9

a

t[2]

R2X[1]=0.492
R2X[2]=0.171
Q2=0.563

t[1]

b

t[2]

R2X[1]=0.065
R2X[2]=0.272
Q2=0.262

t[1]

c

t[2]

R2X[1]=0.221
R2X[2]=0.280
Q2=0.577

t[1]

d

t[2]

R2X[1]=0.509
R2X[2]=0.136
Q2=0.687

t[1]

e

t[2]

R2X[1]=0.564
R2X[2]=0.199
Q2=0.680

t[1]

Healthy adults

Man   Women

Pregnant

Newborn

Primary BAs

CDCA · CA · LCA · DCA · 12-epiDCA

Secondary BAs

UDCA · isoUDCA · UCA · isoUCA · 12-epiCA

Tertiary BAs

HDCA · DCA-5β-ol · DCA-6α-ol · DCA-1β-ol · DCA-4β-ol

Newborn-specific BAs

αHCA · 3-dehydroCA · 12-oxoCDCA · isoCA

Tetra-BA-2 · Tetra-BA-5 · Tetra-BA-6 · Tetra-BA-7 · Tetra-BA-9

Downstream metabolite of CDCA

Downstream metabolite of CA

a

Human bile acids metabolism pathways

Primary BA
Secondary BA
Tertiary BA
Newborn-specific BA

b

CYP3A7-prefering reaction

CYP3A4-prefering reaction

DCA 19-oxidation

DCA 4β-oxidation

DCA 6α-oxidation

DCA 1β-oxidation

DCA 5β-oxidation

DCA 6β-oxidation

CL_{int} (mL/min/μg protein)

Donor age