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# Changes in conjugated urinary bile acids across age groups

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ABSTRACT

Bile acid compositions are known to change dramatically after birth with aging. However, no reports have described the transition of conjugated urinary bile acids from the neonatal period to adulthood, and such findings would noninvasively offer insights into hepatic function. The aim of this study was to investigate differences in bile acid species, conjugation rates, and patterns, and to pool characteristics for age groups. We measured urinary bile acids in spot urine samples from 92 healthy individuals ranging from birth to 58 years old using liquid chromatography tandem mass spectrometry (LC/ESI-MS/MS). Sixty-six unconjugated and conjugated bile acids were systematically determined. After birth, urinary bile acids dramatically changed from fetal (i.e., $\Delta^4$-, $\Delta^5$-, and polyhydroxy-bile acids) to mature (i.e., CA and CDCA) bile acids. Peak bile acid excretion was 6–8 days after birth, steadily decreasing thereafter. A major change in bile acid conjugation pattern (taurine to glycine) also occurred at 2–4 months old. Our data provide important information regarding transitions of bile acid biosynthesis, including conjugation. The data also support the existence of physiologic cholestasis in the neonatal period and the establishment of the intestinal bacterial flora in infants.

## 1. Introduction

A 1987 study revealed excretion of a large amount of unusual bile acids in the urine of individuals with inborn error of bile acid synthesis (IEBAS) [1], demonstrating potential value using the determination of urinary bile acids as a diagnostic tool. Since then, urinary bile acid analysis has been widely used for IEBAS screening in children. As bile acids are present in conjugated forms (i.e., taurine [T], glycine [G], and/or 3-sulfates) at much lower concentrations in urine than in blood, detailed analyses have commonly been conducted by gas chromatography (GC) or GC-mass spectrometry (GC/MS) after group separation of the conjugate forms using anion exchange resin [2]. A significant amount of time is thus required for pretreatment and analysis. Only a small number of pediatric studies using this method have been reported. Kimura *et al* analyzed urinary bile acids in 66 healthy individuals from birth to adulthood by GC/MS and reported significant changes in levels of $\Delta^4$-3-oxo bile acids and polyhydroxylated bile acids during the first month after birth [3]. Takei *et al* analyzed urinary bile acids in 337 samples from healthy individuals including children and adults. By comparing levels of unusual bile acids in IEBAS and hepatic

*Abbreviations: GC*, gas chromatography; *GC/MS*, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; IEBAS, inborn error of bile acid synthesis; *LC/ESI-MS/MS*, liquid chromatography-electrospray ionization tandem mass spectrometry; Cre, creatinine; TBA, total bile acid; taurine, T, glycine: G.

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## 2. Experiment

### 2.1. Subjects

Ninety-two healthy Japanese subjects from whom we were able to collect urine at various time points were used for this study: 6 cases (6 males) for 0 days old, 7 cases (7 males) for 3–4 days old, 4 cases (3 males, 1 female) for 6–8 days old, 8 cases (5 males, 3 females) for 1 month old, 7 cases (4 males, 3 females) for 2–4 months old, 8 cases (4 males, 4 females) for 5–7 months old, 7 cases (4 males, 3 females) for 11–12 months old, 8 cases (4 males, 4 females) for 2–3 years old, 5 cases (3 males, 2 females) for 9–14 years old, 16 cases (6 males, 10 females) for 19–37 years old, and 16 cases (7 males, 9 females) for 40–58 years old. Randomly timed urine samples were collected from subjects and stored at −20 °C until analysis. In all cases, urinary creatinine (Cre) was measured by the Jaffe method using a spectrophotometer [8]. Urinary bile acid concentrations are expressed as millimoles per mole of creatinine (mmol/mol Cre) [9]. None of the subjects had any history or showed signs of hepatobiliary or gastrointestinal disease. Infants were only breast-fed until about 6 months old. Statistical significance was calculated for those age groups with adequate sample size (two-tailed paired or unpaired *t*-test). No sex differences were identified in total bile acid (TBA), composition, or conjugation rate in any age group.

The study protocol was reviewed and approved by the ethics committee at Juntendo University (approval number 16–191, 24–513). Written informed consent was obtained from parents of neonates and children, and from adults themselves. The study was also in compliance with the 1964 Declaration of Helsinki and its later amendments (as revised in Edinburgh 2000) or comparable ethical standards.

### 2.2. Materials and reagents

Abbreviations and trivial names of bile acids used in this study are shown in Supplementary Table S1. Standard products such as CA, CDCA, UDCA, DCA, LCA, HCA, GCA, GCDCA, GUDCA, GDCA, GLCA, GHCA, TCA, TCDCA, TUDCA, TDCA, TLCA, and THCA were purchased from Sigma Chemicals (St. Louis, MO), while [2,2,4,4-$d_4$]-CA ($d_4$-CA, internal standard (IS) for unconjugated bile acids), [2,2,4,4-$d_4$]-GCA ($d_4$-GCA, IS for glycine-conjugated bile acids), and [2,2,4,4-$d_4$]-TCA ($d_4$-TCA, IS for T conjugated and double-conjugated bile acids) were obtained from CDN Isotopes (Quebec, Canada). CA-3S, CDCA-3S, UDCA-3S, DCA-3S, LCA-3S, GCA-3S, GCDCA-3S, GUDCA-3S, GDCA-3S, GLCA-3S, TCA-3S, TCDCA-3S, TUDCA-3S, TDCA-3S, and TLCA-3S were synthesized using previously reported methods [10,11]. GCA-1β-ol, TCA-1β-ol, CA-1β-ol, GCA-6α-ol, TCA-6α-ol, CA-6α-ol, and CDCA-1β-ol were synthesized by a previously reported method [12]. CA-$\Delta^4$-3-one, GCA-$\Delta^4$-3-one, TCA-$\Delta^4$-3-one, CDCA-$\Delta^4$-3-one, GCDCA-$\Delta^4$-3-one, TCDCA-$\Delta^4$-3-one, DCA-$\Delta^{4,6}$-3-one, and LCA-$\Delta^{4,6}$-3-one were provided by Professor Iida (Nihon University, Tokyo Japan). Synthesis of $\Delta^5$-3β-ol, $\Delta^5$-3β,7α-diol, $\Delta^5$-3β,7α,12α-triol, $\Delta^5$-3β-ol-3S, $\Delta^5$-3β,7α-diol-3S, $\Delta^5$-3β,7α,12α-triol-3S, G$\Delta^5$-3β-ol, G$\Delta^5$-3β,7α-diol, G$\Delta^5$-3β,7α12α-triol, G$\Delta^5$-3β-ol-3S, G$\Delta^5$-3β,7α-diol-3S, G$\Delta^5$-3β,7α12α-triol-3S, T$\Delta^5$-3β-ol, T$\Delta^5$-3β,7α-diol, T$\Delta^5$-3β,7α12α-triol, T$\Delta^5$-3β-ol-3S, T$\Delta^5$-3β,7α-diol-3S, and T$\Delta^5$-3β,7α12α-triol-3S was performed using a previously reported method [13]. Ethanol, methanol, acetonitrile, water, and formic acid were of LC/MS grade, ammonium acetate was of analytical grade, and all were purchased from Kanto Chemical Co. Ltd. (Tokyo, Japan).

### 2.3. Preparation of standards

Stock solutions of the 66 bile acids were prepared separately at 10 μmol/mL in ethanol and stored at −20 °C. For the preparation of calibration standard solutions, equal volumes of stock solutions were mixed and the mixtures were diluted to 10, 30, 100, 300, 1000, and 3000 pmol/mL with 50% ethanol. Prepared calibration standard solutions were stable in analytical glass vials for 4 weeks at 4 °C. For IS solutions, a mixture of $d_4$-CA, $d_4$-GCA, and $d_4$-TCA (100 nmol/mL for each BA) was prepared in the same manner.

### 2.4. Preparation of samples

After thawing, 10 μL of IS was added to 100 μL of the urine sample. The solution was quickly transferred onto a solid-phase extraction cartridge (InertSep C18-B 100 mg/1 mL; Agilent Technologies Japan, Tokyo) that had been pre-conditioned with 1 mL of methanol and 3 mL of $H_2O$. After loading the sample, the column was washed with 1 mL of $H_2O$, then the desired bile acids were eluted with 1 mL of 90% ethanol. After evaporation of the solvent under an $N_2$ stream at 40 °C, the residue was dissolved in 1 mL of 50% ethanol, then 20 μL of the solution was immediately analyzed by LC/ESI-MS/MS as described below.

### 2.5. LCI/ESI-MS/MS conditions

A TSQ Quantum Discovery Max mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an electrospray ionization probe was used. A Surveyor HPLC system (Thermo Fischer Scientific) was used for chromatographic separation. The column was an InertSustain C18 (150 mm × 2.1 mm internal diameter, 3 μm particle size; GL Sciences, Tokyo, Japan) employed at 40 °C. A mixture of 5 mM ammonium acetate and acetonitrile was used as the eluent at a flow rate of 0.2 mL/min. Gradient elution was programmed as follows: 0–0.5 min, 10% acetonitrile (constant); 0.5–5 min, 10–22% acetonitrile (linear); 5–36 min, 22–60% acetonitrile (linear); 36–46 min, acetonitrile 60–98% (linear); 46–50 min, 98% acetonitrile (constant). Total run time was 50 min. Negative ion electrospray parameters were set as follows: The collision gas (argon) pressure and the collision energy (argon) pressure were kept at 1.3 mm Torr and 27–55 eV, respectively. Selective reaction monitoring transitions and collision energy exploited for each bile acid and IS, as well as their retention times, are shown in Supplementary Table S2. Our previous reports [6,13] were used as references for the identification and quantification of individual bile acids, including relative retention times with respect to the IS and their base peak ions.

### 2.6. Method validation

For accuracy and precision studies, 20, 200, or 2000 pmol of standard bile acids were spiked into healthy urine (1 mL). Recovery rate was calculated as [(post-spiked urine concentration – pre-spiked urine concentration)/spiked concentration] × 100 (%), and precision was obtained in terms of the coefficient of variation (RSD, %; n = 5).

### 2.7. Stability testing for urinary bile acids

For testing stability of urinary bile acids, standard bile acids (500 pmol) were spiked into healthy urine (1 mL). This spiked urine was left to stand at different temperature (-20 °C, +4°C, +25 °C, and + 37 °C) until 4 weeks. Stability rate (%) was calculated as [concentration after the passage/initial concentration] × 100 (%). To study prolonged stability (up to 3 years), urine specimens preserved at −20 °C were re-analyzed (n = 4). Recovery rate (%) was calculated as [observed concentration/concentration obtained from analysis of the fresh specimen] × 100 (%).

### 2.8. Statistics

All data are reported as mean ± standard deviation in the table. One-way analysis of variance was used to determine the significance of differences between groups. Groups were compared using Bonferroni/Dunn analysis. Values of $p < 0.05$ were accepted as statistically significant.

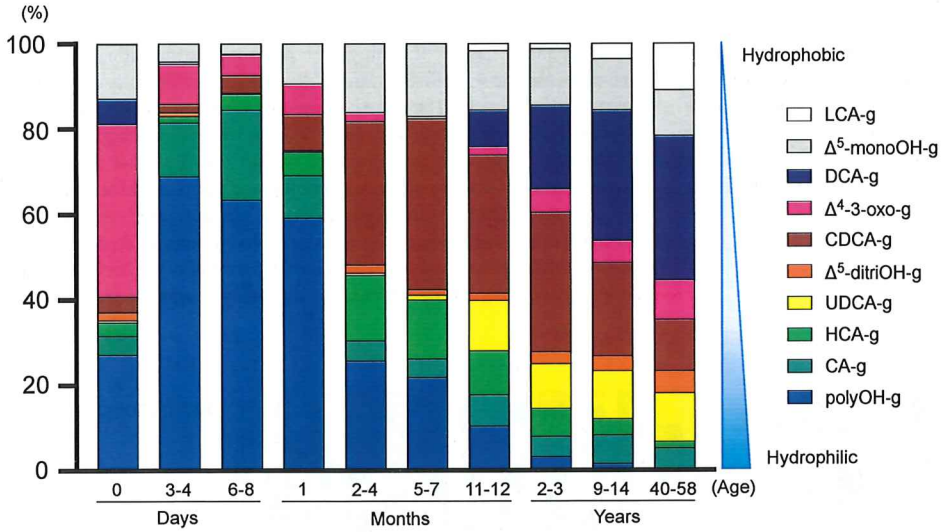


**Fig. 2.** Change in bile acid composition with age by backbone-originated structure. At birth, $\Delta^4$-3-oxo-g was predominant (40%), followed by the polyhydroxylated form (27%) and $\Delta^5$-g (15%), indicating that about 80% of total urinary bile acids were fetal bile acids. The primary bile acids, CA-g and CDCA-g, were present almost equally at birth (~4%), with CDCA-g increasing from around 2–4 months old and becoming predominant. Secondary bile acids (DCA and LCA) were observed from around 11–12 months old. Bile acid groups are shown in approximate order of hydrophobicity, from more hydrophobic groups (upper) to more hydrophobic groups (bottom).

(approximately 12% of TBA), then gradually decreased thereafter. HCA-g, the 6α-hydroxylated metabolite of CDCA, presented throughout all age groups for which composition was relatively higher from the neonatal through infant periods. Peak concentration of this bile acid group was 1.4 mmol/mol Cre (4% of TBA) at 6–8 days old. In the adult specimens (19–37 years old), HCA-g was barely detectable (<1% of TBA).

With regard to secondary bile acids, LCA-g was barely detectable at birth. Slight DCA-g was found at birth (0.23 mmol/mol Cre, 6% of TBA), but quickly disappeared by 6–8 days old. This suggests that these secondary bile acids were of maternal origin. LCA-g and DCA-g levels increased significantly as of 11–12 months old and were established as 0.04 (10% of TBA) and 0.14 mmol/mol Cre (10% of TBA) in adulthood, respectively.

### 3.3. Changes in conjugation patterns of bile acids with age

Fig. 3 presents changes in conjugated and unconjugated bile acid patterns by age groups. Approximately 65% of bile acids presented as the conjugated forms at birth, constituting 32% of taurine (T) forms, 8% of glycine (G) forms, 3% of 3-sulfate (S) forms, and 22% of double-conjugated forms (TS and GS forms). Interestingly, as much as 35% of bile acids presented as unconjugated forms at birth. This percentage of unconjugated bile acids was highest across all age groups. Proportions of T and G mono-conjugated forms quickly elevated after birth, and these two forms accounted for approximately 90% of TBA at 6–8 days old. In neonates, the *T*-conjugated form predominated over the G-conjugated form. Conjugation with T renders a bile acid more hydrophilic than conjugation with G, which allows the body to more easily excrete the conjugated bile acid into urine early in life. Although a high proportion of the G mono-conjugated form (approximately 44%) was found at 6–8 days old, this quickly declined with the increase in the more hydrophilic GS double-conjugated form. In adults, most G-conjugated bile acids were excreted as the GS double-conjugated form. The TS double-conjugated form was constantly found at low levels throughout all age groups.

A clear change in conjugation pattern was found at 2–4 months old. As of this age, G and GS forms were markedly elevated with reduced composition of the *T*-conjugated form. These G-conjugated and GS forms became the major conjugation forms later in life. Only small amounts of 3-mono S were observed in any age group, with a level of only 0.5–2% in adulthood. This may imply that 3-sulfation alone does not result in a

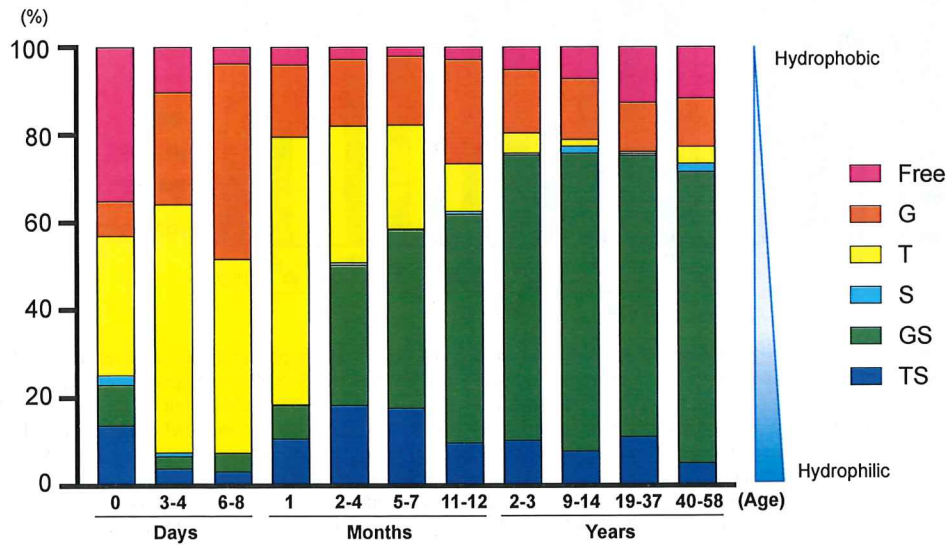


**Fig. 3.** Changes in conjugation patterns of bile acids by age group. At birth, the conjugated type in total bile acids accounts for 65%, with *T*-conjugates predominating (32%). At 6–8 days old, G-conjugate increased and continued to be observed at around 10% until adulthood. GS double-conjugate increased at 2–4 months old and became the main conjugation type until adulthood. Abbreviations: TS, taurine at C-24 and sulfate at C-3 conjugated bile acids; GS, glycine at C-24 and sulfate at C-3 conjugated bile acids; S, free at C-24 and sulfate at C-3 conjugated bile acids; T, taurine at C-24 conjugated and free at C-3 bile acids; G, glycine at C-24 conjugated and free at C-3 bile acids; Free, free at C-24 and free at C-3 bile acids. Bile acid groups are shown in approximate order of hydrophobicity, from more hydrophobic groups (upper) to more hydrophobic groups (bottom).

Instead, the TS double-conjugated type was the most abundant at birth, accounting for 50%. The TS double-conjugated form steadily decreased after 3–4 days old, with little found in adult specimens. With the shift from T to G conjugation with age (Fig. 3), the rate of the GS double-conjugated form steadily increased to become the main conjugation type after 6–8 days.

Tri- and tetra-hydroxy acids are categorized in the polyOH-g group. This group of bile acids is the most hydrophilic of the groups analyzed in this study. As expected, the T mono-conjugated form represented>80% at birth and continued to be the most abundant type until 11–12 months. At 1 year after birth, G conjugation started replacing the *T*-conjugated form and became the main conjugation form. Unlike other groups of bile acids, almost 100% of polyOH-g was found to be G mono-conjugated.

The $\Delta^5$-monoOH-g group of bile acids has the base structure of 3β-hydroxy-5-cholenoic acid. This unsaturated mono-hydroxy bile acid has been shown to be both very hydrophobic and hepatotoxic, and must therefore be efficiently excreted into urine. As with other hydrophobic bile acids, the TS double-conjugated type was most abundant at birth, then decreased later in life. The GS double-conjugated type increased and became the main conjugation type after 5–7 months old.

Unlike other groups of bile acids, the $\Delta^4$-3-oxo group of bile acids lacks a 3α-hydroxyl group, which is a site for sulfation. Amination is therefore the only way to increase water solubility for $\Delta^4$-3-oxo bile acids, and seems key to efficient renal secretion of this group of bile acids. Surprisingly, the non-amidated (unconjugated) form was most frequently observed in all age groups except at 1 month, when the T conjugation dominated (84%). The T conjugation was observed until 2–4 months old, then the G-conjugated form constituted approximately 24% thereafter. The non-amidated and G-conjugated forms were mainly observed in adults.

### 3.5. Method validation and stability studies

With the described LC-MS/MS conditions, simultaneous separation and determination of all 66 targeted bile acids was achieved within 50 min. This method provides sufficient linearity for all urinary bile acids tested in this study, and analysis can be performed within a 300-fold dynamic range with good correlations ($r > 0.98$) (Supplementary Table S2). The detection limit (signal-to-noise ratio $\geq 5$) was estimated as 0.03–3.18 pmol/mL for all targeted bile acids (data not shown). Assay validation data are provided in Supplementary Tables S3 and S4, demonstrating that the method allows practical determination of common human urinary bile acids within the range of 20–2000 pmol/mL. Stability data for individual bile acids in urine are also presented in Supplementary Table S5. Most bile acids were stable for the initial 4 days when the specimen was stored at < 4 °C. However, after 1 week, some bile acids such as $\Delta^5$-mono-g and LCA-g showed gradual decomposition at 4 °C. At −20 °C, most bile acids were stable for 1–3 years (recovery > 85%), with the exception of $\Delta^4$-3-oxo-g bile acids, which showed a recovery rate of 72–77% after 1 year of storage.

## 4. Discussion

With aging, dramatic changes occur in the concentration and hydrophobicity of urinary bile acids. More specifically, characteristic changes in bile acid metabolism in newborns and infants suggest protective hepatic metabolic changes in the bile acid pool as the neonatal liver transitions from a predominantly acidic pathway to a neutral pathway, with elimination of bile acids of maternal origin, and with correction of the described physiologic cholestasis. Possible causes of physiologic cholestasis include decreased excretion of maternal and neonatal bile acids from the liver due to the immaturity of bile acid transporters [14], effects of enterohepatic circulatory enhancement resulting from frequent feeding [15], and bile acid transition from the portal vein to systemic circulation by the physiologic persistence of venous ducts.

The present study showed that levels of urinary TBA dramatically increased after birth and peaked at 6–8 days old. This trend agrees with results from a GC/MS analysis by Kimura *et al* [3]. Various fetal bile acids are thus detected in urine during this period [12]. In our analysis, approximately 40% of $\Delta^4$-3-oxo-g, 27% of polyOH-g, and 13% of $\Delta^5$-monoOH-g bile acids were found at birth, indicating that > 80% of TBAs were fetal bile acids. Presence of $\Delta^4$-3-oxo-g and $\Delta^5$-monoOH is likely due to the reduced physiologic activity of 5β-reductase in neonates [16,17]. Notably, secretion of bile is considered to initiate at approximately 4 months of gestation [18]. However, bile acids are reportedly already present in the fetal gallbladder bile from 14 to 20 weeks of gestation, with compositions higher in CDCA than in CA [19]. This evidence also supports the predominance of the acidic pathway in the fetal liver. Physiologic cholestasis in early neonates is normally corrected by additional hydroxylation to common bile acids in the liver, decreasing hydrophobicity thus lessening toxicity and facilitating renal excretion of bile acids [16,20]. The 1β-hydroxylation is considered as the most common excretion-promoting mechanism against hypercholanemic disorder [16]. *T*-conjugated 1β-hydroxy CA (TCA-1β-ol) is highly water-soluble and is thus efficiently excreted into urine during this period. All fetal bile acids gradually decreased with age. The observation of $\Delta^4$-3-oxo-g and $\Delta^5$-monoOH-g bile acids in adult specimens was interesting (Figs. 2, 4), suggesting that acidic pathways remain active to some degree even in the mature liver. The physiologic roles of these bile acids in adult life are unclear and warrant investigation. The more predominant role of bile acid synthesis in early life has been proposed to regress to the true function of the acidic pathway, which is the formation of key regulatory oxysterols [21,22].

Conjugation of 24-amino-*N*-acyl with T or G [23], 3α-sulfation [24], and 7β-*N*-acetylglucosamine (NAG) of UDCA [25,26] are common conjugated forms for bile acids. These conjugated bile acids show increased water solubility and are more likely to be excreted into urine. Amino-*N*-acyltransferase is responsible for bile acid conjugation with T and G [27]. Amidated bile acids are then excreted into bile by the bile salt export pump (BSEP) on the canalicular membrane of hepatocytes [14], and facilitate micellization to absorb fat from the intestine. In adults, the conjugation ratio of G to T depends on taurine intake, and the reason for the predominance of T conjugation at birth remains unclear [15]. In the human liver, bile acids can also be sulfated [28]. SULT2A1 is the major enzyme shown to have sulfotransferase activity for bile acids [29]. In rats, sulfotransferase activity is observed not only in the liver, but also in the kidney [30]. Sulfation significantly increases the hydrophilicity of bile acids, facilitating renal excretion. Our data show that renal excretion of sulfated bile acids was markedly elevated at 2–4 months old, with an increase in G-conjugated forms. This observation may indicate that SULT2A1 activity is significantly elevated at 2–4 months to facilitate the secretion of hydrophobic G-conjugated bile acids. Differences in SULT2A1 activity have been also reported by sex [30], but no such sex differences were evident in urinary rates of sulfated bile acids at any age group in the present study.

In the colon, conjugated bile acids are de-conjugated and 7α-dehydroxylated by bacterial bile salt hydrolase (BSH) [31]. As a result, CA and CDCA are converted to DCA and LCA, respectively. A previous report showed that 7α-dehydroxylation bacteria (i.e., *Clostridium*, *Lactobacillus*, *Bacteroides*, and *Eubacterium* species) start colonization and proliferation in the intestine when supplementation with baby food begins [32,33]. We have previously analyzed samples of meconium [16] and feces from infants up to 1 month old (unpublished data). Those data revealed that DCA and LCA gradually decreased after birth and had almost disappeared by 28 days old. In addition, Hammons *et al* reported significant elevations of fecal DCA and LCA around 26–27 weeks [34]. In our urinary analysis, the maternal origin of DCA and LCA quickly disappeared after birth and reappeared around 10 months (=40 weeks), several weeks after the significant elevations of fecal DCA and LCA reported by Hammons *et al* [34]. Once the bacterial flora becomes established, secondary bile acids are not well absorbed from the large

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