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Increased Serum Concentrations of Secondary Bile Salts during Cholate Feeding Are Due to Coprophagy. A Study with Wild-Type and Atp8b1-Deficient Mice

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Abstract: Coprophagy (i.e., consumption of feces) is a behavior seen in rodents and other animal species. This behavior can substantially influence the enterohepatic cycling of compounds, including bile salts. Since many studies involve the feeding of rodents with bile salt supplemented diets, it is of importance to know the influence of coprophagy on bile salt composition in such studies. We compared the peripheral and portal bile salt composition of mice in conventional and metabolic cages when fed a control diet or a diet containing 0.5% cholate. We also performed these experiments with Atp8b1-deficient mice as it has been suggested that in the absence of this transporter bile salt absorption in the intestine would be increased. In mice on a control diet there is little difference in bile salt composition between conventional housing and metabolic housing. Metabolic housing led to a near complete disappearance of the low levels of dihydroxy bile salts (i.e., deoxycholate + chenodeoxycholate) in peripheral serum. In mice fed a control diet, the portal blood concentration of unconjugated dihydroxy bile salts was extremely low (<2%), but these rose to about 10% when mice were fed a cholate-supplemented diet. In metabolic cages the portal blood content of these unconjugated dihydroxy bile salts was reduced to undetectable levels. Whether housed in conventional cages or in metabolic cages, wild-type and Atp8b1-deficient mice had similar concentrations in portal blood, suggesting that intestinal bile salt absorption is not altered in Atp8b1-deficient mice.

Keywords: Atp8b1; bile salts; coprophagy; mouse; bile acids

Introduction

Several species such as rats,[1] chimpanzees,[2] and birds[3] are known to eat their own feces, which is called coprophagy. Coprophagy can have a great effect on metabolism of different compounds, including bile salts. The majority of bile salts (>95%) is reabsorbed in the distal part of the ileum, by the apical sodium-dependent bile acid transporter, Asbt.[4−6] In the large intestine, nonabsorbed bile salts are unconjugated

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and dehydroxylated by intestinal bacteria to secondary bile salts, such as deoxycholate and lithocholate.[7] Coprophagy leads to the ingestion of these secondary bile salts, which will re-enter the circulation via the ileal bile salt transporter.

In order to determine if coprophagy determines the bile salt pool composition, we housed mice in conventional cages or in metabolic cages. In conventional but not metabolic cages, the mice were able to consume their own feces. We have determined the bile salt concentration and composition of peripheral and portal blood in these mice.

The mice used in these experiments were wild-type and *Atp8b1*$^{G308V/G308V}$ mice. The latter is a model for progressive familial intrahepatic cholestasis type 1 (PFIC1). Patients with this disease develop cholestasis followed by end-stage liver disease before adulthood.[8] Chen et al.[9] have provided evidence that ASBT is overexpressed in these patients and that this may lead to enhanced bile salt absorption in the terminal ileum. In line with this suggestion we have observed that *Atp8b1*$^{G308V/G308V}$ mice on a 0.5% cholate diet accumulate very high concentrations of bile salts in the circulation. Concomitant with this bile salt accumulation these mice have elevated serum transaminase levels, indicating damage to the liver.[10] We therefore investigated whether bile salt absorption is enhanced in these animals.

Materials and Methods

Animals. All experiments were performed with age-matched male mice at 2−6 months of age. Wild-type and *Atp8b1*$^{G308V/G308V}$ mice used in the experiments were of 129/SvImJ background and bred at the animal institute of the Academic Medical Center, Amsterdam.[10] Mice were fed a commercial purified diet (K4068.02, Arie Blok Diervoeders, Woerden, The Netherlands), either supplemented with 0.5% w/w sodium cholate (Merck, Darmstadt, Germany) or not supplemented. Food and water were supplied ad libitum. Mice were maintained on a 24 h light−dark cycle and weighed each morning. Metabolic housing means that the mice were housed per two on a metal grate. In this setup the mice cannot eat feces because the feces (and urine) fall through the grate. The mice are, however, still able to eat the feces directly from their cagemate. Weighing of the animals was done every morning in a 24 h cycle. The animals were sacrificed at approximately the same time (10.00 a.m.) in the light−dark cycle, 7 days after the start of the feeding experiment. All animal experiments were approved by the institutional animal care and use committee (IACUC) of the Academic Medical Center.

Portal Blood Collection. After being fed a control or cholate-supplemented diet for 7 days, the mice were anesthetized by intraperitoneal injection of 1 mL/kg Hypnorm (fluanisone/fentanylcitrate) and 10 mg/kg diazepam. Blood was collected by orbital punction as a measure for peripheral blood. The vena cava caudalis was injected with 50 μL of heparin (1000 units/mL). The portal vein was cannulated, and blood was collected for 5 min. Serum was prepared by centrifugation (4000 rpm, 10 min), and 20 μL was used to determine the bile salt composition by HPLC/MS.[11] Using this procedure no distinction could be made between deoxycholate and chenodeoxycholate.

Determination of Aminotransferases in Serum. Serum aminotransferase enzyme activities were determined at the routine clinical chemistry laboratory.

Statistics. The reported data are given as means ± SD. Significance was tested by use of the one-way ANOVA or the two-way ANOVA with Bonferroni's correction for multiple testing.

Results

Body Weight and Serum Transaminases. The mice were weighed every day during the 7 days on the specified diet. The weight on day 7 is given as the percentage of the weight on day 0 (Figure 1). A significant decrease in body weight was seen for the *Atp8b1*$^{G308V/G308V}$ mice on the cholate diet, both in the conventional and in the metabolic cage.

Aminotransferases AST (Figure 2A) and ALT (Figure 2B) were measured in peripheral serum to assess the damage to the liver. Both did not differ between conventional or metabolic housing. As previously reported,[10] serum transaminases were increased in *Atp8b1*$^{G308V/G308V}$ mice (but not in wild-type mice) on a cholate diet. This induction was similar in conventional and metabolic cages.

Total Serum Bile Salt Levels in Wild-Type and Atp8b1-Deficient Mice. Figure 3 shows the total bile salt concentration in peripheral (panel A) and portal (panel B) serum, as

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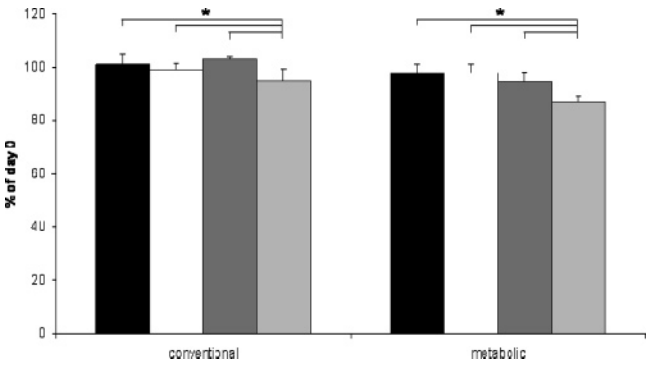

Figure 1. Mouse body weights after 7 days of treatment. The weights on day 7 are depicted as percentage of the weight on day 0. Black bars: wild type, control diet. White bars: *Atp8b1*$^{G308V/G308V}$, control diet. Dark gray bars: wild type, 0.5% cholate diet. Light gray bars: *Atp8b1*$^{G308V/G308V}$, 0.5% cholate diet. Conventional housing: $n = 7$. Metabolic housing: $n = 6$. Significance was tested by use of the two-way ANOVA with Bonferroni's correction for multiple testing. (∗) $p < 0.05$.

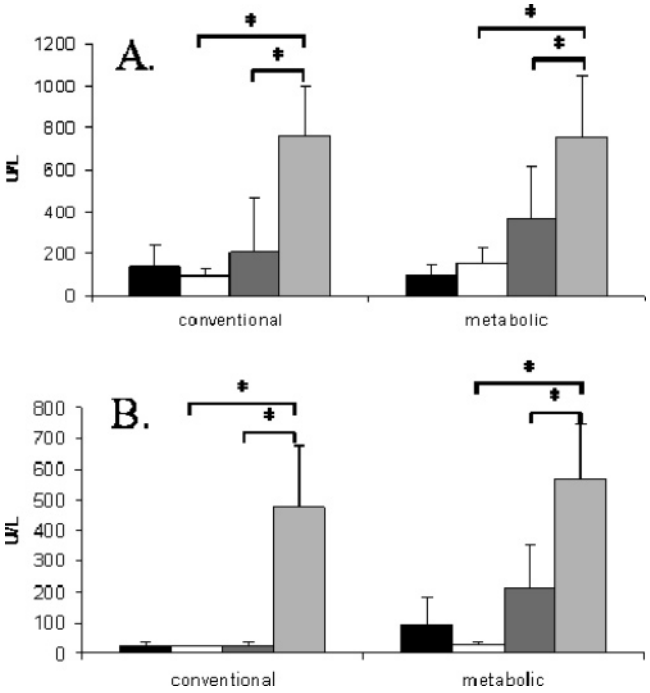

Figure 2. Aminotransferase activity in serum as an indicator of liver damage AST (A) and ALT (B) levels present in serum of wild-type and *Atp8b1*$^{G308V/G308V}$ mice. Black bars: wild type, control diet. White bars: *Atp8b1*$^{G308V/G308V}$, control diet. Dark gray bars: wild type, 0.5% cholate diet. Light gray bars: *Atp8b1*$^{G308V/G308V}$, 0.5% cholate diet. Conventional housing: $n = 7$. Metabolic housing: $n = 6$. Significance was tested by use of the two-way ANOVA with Bonferroni's correction for multiple testing. (∗) $p < 0.05$.

well as the difference between these two (panel C). *Atp8b1*$^{G308V/G308V}$ mice had a high peripheral serum bile salt level on the cholate diet. In the metabolic cage, this tended to be less than in the conventional housing, but this difference did not reach significance. In both wild-type and *Atp8b1*$^{G308V/G308V}$ mice on a cholate diet, the difference

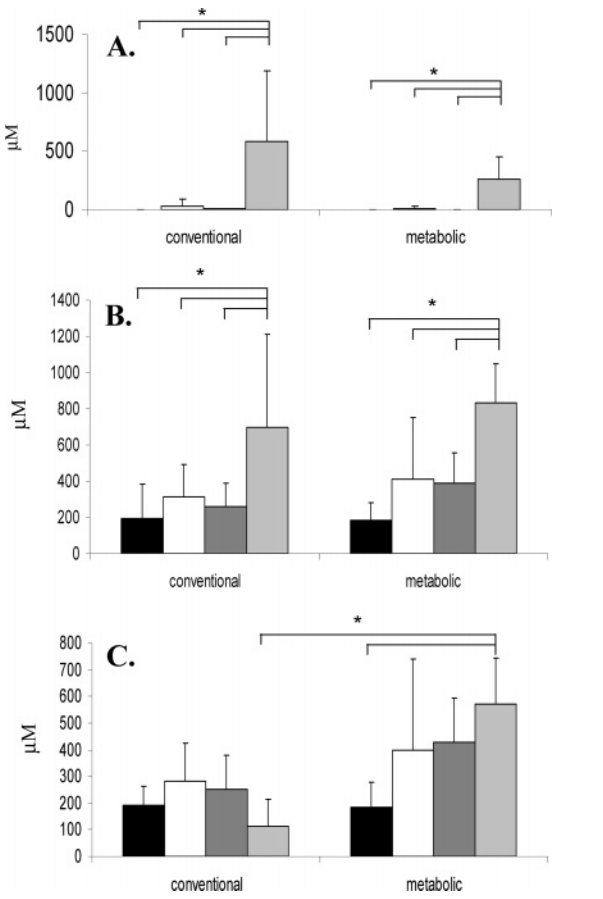

Figure 3. Bile salt concentrations in peripheral and portal serum: (A) peripheral serum values in μM, (B) portal serum values in μM, and (C) difference between portal and peripheral values, calculated per mouse and then averaged. Black bars: wild type control diet. White bars: *Atp8b1*$^{G308V/G308V}$ control diet. Dark gray bars: wild type 0.5% cholate diet. Light gray bars: *Atp8b1*$^{G308V/G308V}$ 0.5% cholate diet. Conventional housing: $n = 7$. Metabolic housing: $n = 4-6$. Significance was tested by use of the two-way ANOVA with Bonferroni's correction for multiple testing. (∗) $p < 0.05$.

between peripheral and portal bile salt concentration was larger in the metabolic housing than in conventional housing. This difference reached significance in *Atp8b1*$^{G308V/G308V}$ mice.

Peripheral Bile Salt Composition. Using HPLC−tandem MS we also analyzed the bile salt species in peripheral and portal serum. With this method we could not distinguish between deoxycholate (DCA) and chenodeoxycholate (CDCA), which together are referred to as dihydroxy (di-OH) bile salts. In our experience mice have little if any chenodeoxycholate. Figure 4A shows the peripheral bile salt composition of wild-type mice on conventional vs metabolic housing. This figure shows that the most prominent bile salts were taurocholate and tauromuricholate. Metabolic housing reduced the trace amounts of tetrahydroxy bile salt and unconjugated ursodeoxycholate and dihydroxy bile salts.

As reported previously, *Atp8b1*$^{G308V/G308V}$ mice on a control diet had slightly but significantly higher serum bile salt

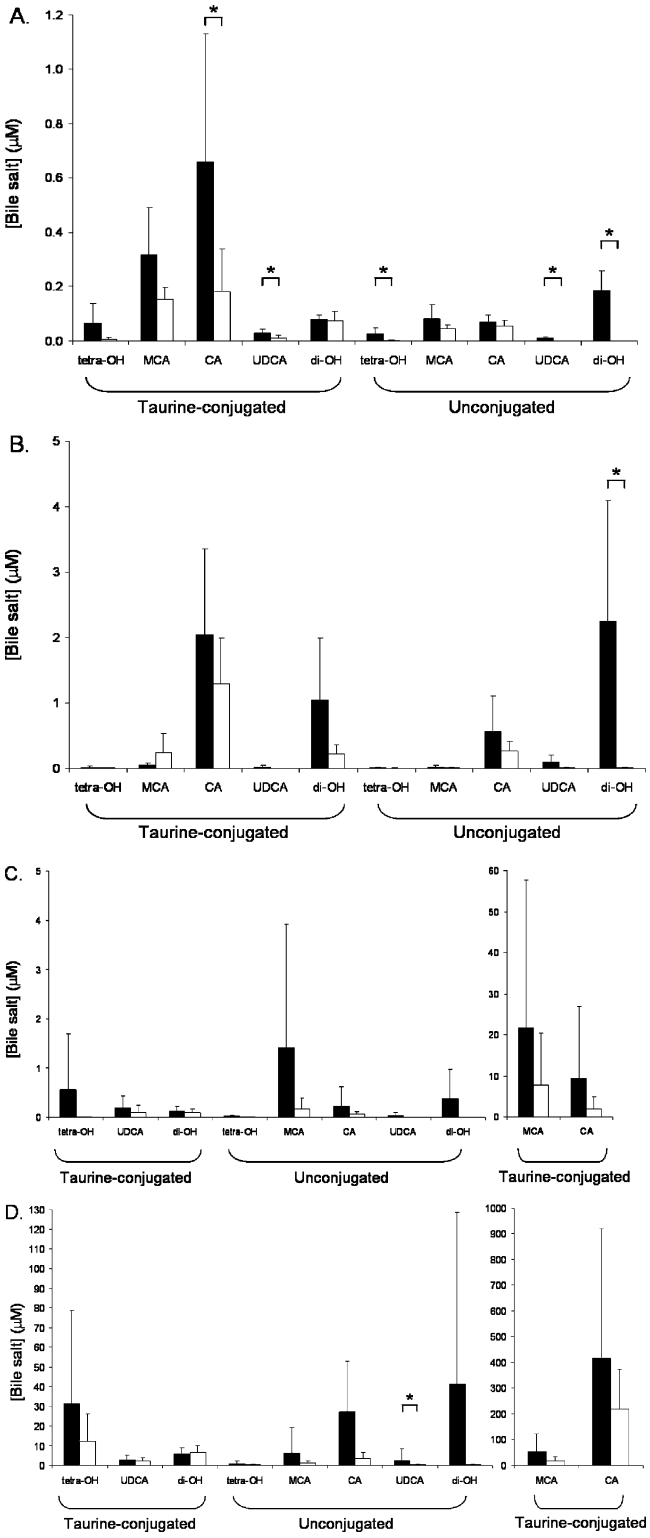

Figure 4. Concentrations of bile salt species in peripheral serum. Peripheral serum was analyzed for bile salt species: tetra-OH, tetrahydroxyl bile salt; MCA, muricholate; CA, cholate; UDCA, ursodeoxycholate; di-OH, chenodeoxycholate/deoxycholate. (A) Wild type, control diet (B) Wild type, 0.5% cholate diet. (C) *Atp8b1*$^{G308V/G308V}$, control diet (D) *Atp8b1*$^{G308V/G308V}$, 0.5% cholate diet. Black bars: conventional housing ($n = 7$). White bars: metabolic housing ($n = 4-6$). Significance was tested by use of one-way ANOVA. (*) $p < 0.05$.

concentrations with relatively more tauromuricholate than wild-type mice (Figure 4C). In these mice metabolic housing had the same effects as in wild-type mice (Figure 4C).

Figure 4B shows the same analysis for wild-type mice on a cholate diet. The main difference between animals in conventional and metabolic cages was the near complete disappearance of dihydroxy bile salts from the peripheral serum (2.3 μM to 0.012 μM, respectively). Trace amounts of conjugated ursodeoxycholate that were observed during conventional housing also disappeared in metabolic cages. In *Atp8b1*$^{G308V/G308V}$ mice very high bile salt concentrations were observed on cholate feeding (Figure 4D). In these mice too, a dramatic fall in dihydroxy bile salt levels was observed in metabolic cages (from 41 μM to 0.3 μM). In contrast to wild-type animals, the concentrations of tetrahydroxy bile salt were substantial (>30 μM) in *Atp8b1*$^{G308V/G308V}$ mice, and these did not significantly drop in metabolic cages.

Composition of Bile Salts in the Portal Blood and Correction for Peripheral Blood Content. By subtracting the peripheral blood bile salt concentration from that of portal blood one can estimate the amount of each bile salt species that is carried from the intestine to the liver. This corrected portal bile salt concentration is a relative estimate of intestinal bile salt absorption at that particular point in time. When wild-type mice were fed a control diet, there was little difference between the corrected portal bile salt concentrations in conventional housing versus metabolic cages (Figure 5A). This is also seen for the *Atp8b1*$^{G308V/G308V}$ mice (Figure 5C). Upon cholate feeding, the composition of the absorbed bile salts dramatically changed compared to control diet (Figure 5A vs 5B, black bars). Muricholate (conjugated and unconjugated) dropped to minimal levels while taurocholate became the major bile salt (about 80% of the absorbed bile salt). Importantly, while the corrected portal blood concentration of dihydroxy bile salts was insignificant in mice fed a normal diet (~2 μM, conjugated and unconjugated), dihydroxy bile salts became the second most abundant species upon cholate feeding (~20 μM, for conjugated plus unconjugated). Similar changes were observed in *Atp8b1*$^{G308V/G308V}$ mice (Figure 5C vs 5D, black bars). In metabolic cages the high concentrations of unconjugated dihydroxy bile salts strongly decreased; in wild type they were about 100-fold lower than in conventional cages (Figure 5B) and in *Atp8b1*$^{G308V/G308V}$ mice about 25-fold lower (Figure 5D). Strikingly, the concentrations of conjugated dihydroxy bile salts did not change in metabolic cages. There was no significant difference in the corrected portal blood concentration of total bile salts between wild-type and *Atp8b1*$^{G308V/G308V}$ mice under all conditions tested.

Discussion

Coprophagy is common behavior among many animal species. It is well-known that stools contain large amounts of secondary bile salts, and therefore coprophagy potentially causes a substantial intake of these bile salts. In the present report we demonstrate that the presence of unconjugated dihydroxy bile salts in serum of mice can be nearly

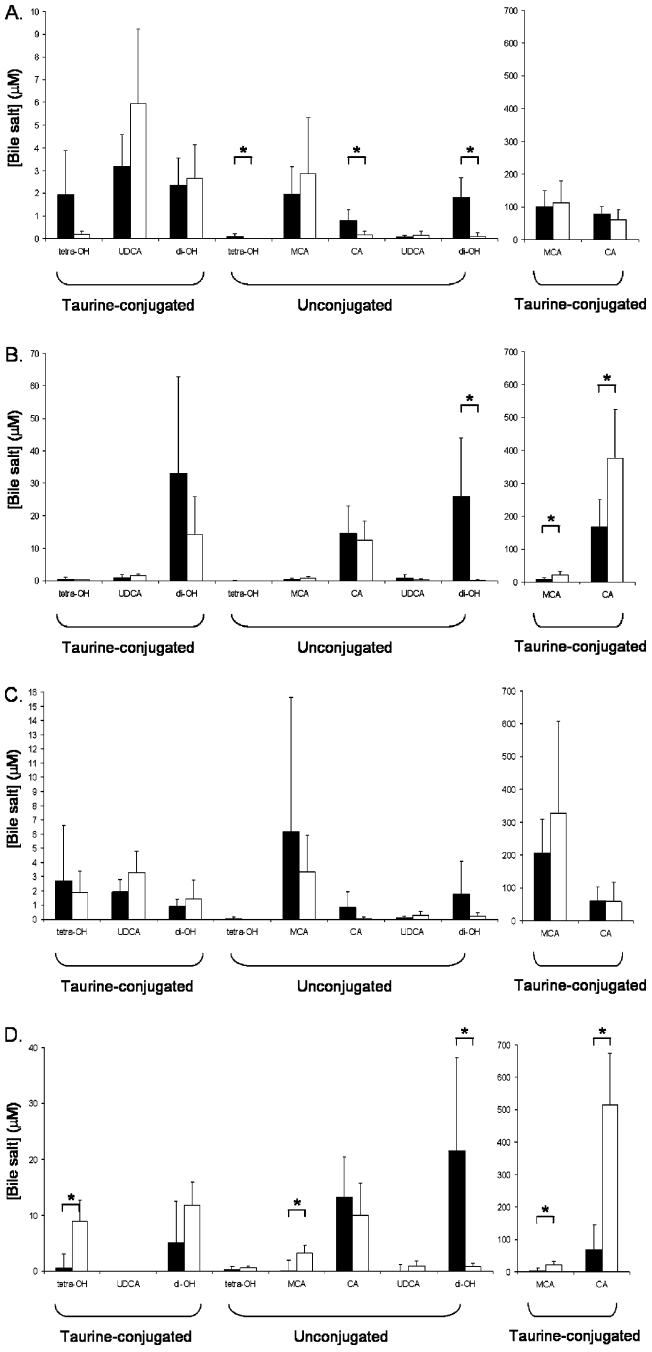

Figure 5. Bile salt composition in portal serum corrected for peripheral serum levels The concentrations of the major bile salt species were determined in peripheral and portal serum. The difference between these values provides an indication of the intestinal bile salt uptake at that particular point in time: tetra-OH, tetrahydroxyl bile salt; MCA, muricholate; CA, cholate; UDCA, ursodeoxycholate; di-OH, chenodeoxycholate/deoxycholate. (A) Wild type, control diet. (B) Wild type, 0.5% cholate diet. (C) *Atp8b1*$^{G308V/G308V}$, control diet. (D) *Atp8b1*$^{G308V/G308V}$, 0.5% cholate diet. Black bars: conventional housing ($n = 7$). White bars: metabolic housing ($n = 4-6$). Significance was tested by use of one-way ANOVA. (*) $p < 0.05$.

completely attributed to coprophagy. This conclusion is based on the fact that the serum concentration of dihydroxy bile salts in mice housed in metabolic cages is extremely low. The applied method of analysis could not distinguish between the dihydroxy bile salts chenodeoxycholate (CDCA) and deoxycholate (DCA), but in our experience mice have little if any CDCA. It should be stressed that the peripheral serum concentration of deoxycholate is a rather insensitive parameter for deoxycholate uptake in the gut, because rodents are extremely efficient in the rehydroxylation of secondary bile salts.[12,13] Portal blood contains all bile salts absorbed by the intestine but has not passed the liver yet. Therefore sampling portal blood provides a more reliable estimate of the amount of secondary bile salt that is being absorbed from the intestine at that particular time point. Strikingly, we observed that portal blood from mice on a control diet contains very little deoxycholate (<2% of the total). Hence, under normal feeding conditions deoxycholate absorption is insignificant. This situation becomes very different under conditions of cholate feeding. We have previously shown that feces of mice on a 0.5% cholate diet contain as much as 50% dihydroxy bile salts.[10] Hence coprophagy will cause substantial intake of these secondary bile salts. Indeed, analysis of portal blood in mice on a cholate diet shows that as much as 20% of the absorbed bile salts consists of deoxycholate (conjugated plus unconjugated). These data show that coprophagy does not substantially influence the bile salt pool under control conditions but leads to considerable absorption of secondary bile salt during cholate feeding. The fact that mice efficiently rehydroxylated deoxycholate most likely explains why the significant increase in deoxycholate absorption leads to only relatively small increases in the peripheral serum concentrations of this bile salt. It must be realized, however, that the liver load of deoxycholate is much higher than would be expected on the basis of peripheral concentrations. Strikingly, the amount of conjugated deoxycholate did not change significantly with metabolic housing. This indicates that conjugated deoxycholate is not derived from coprophagy. Conjugated deoxycholate may be formed in the small intestine from taurocholate and directly taken up.

Progressive familial intrahepatic cholestasis type 1 is an inherited liver disease caused by the absence of ATP8B1 function. ATP8B1 is proposed to function as an aminophospholipid flippase in the apical membrane of various epithelial cell types, including the hepatocyte. How the absence of this function relates to the development of progressive cholestasis is not clear yet. However, recent experiments with *Atp8b1* mutant mice strongly suggest that the cholestatic phenotype is caused by inadequate lipid ordering in the canalicular membrane of the hepatocyte.[17] Ileal tissue from PFIC type 1 patients was found to overexpress the apical bile salt

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transporter ASBT,[9] and both intestinal tissue and liver tissue from these patients have been reported to lack mRNA for the nuclear hormone FXR, which regulates ASBT expression.[9,14] On the basis of these results it was suggested that ATP8B1 is essential for FXR expression and that the lack of FXR signaling leads to overexpression of ASBT and subsequent hyperabsorption of bile salts.[9,14] We have shown that the *Atp8b1*$^{G308V/G308V}$ mouse, an animal model for this disease, accumulates massive amounts of bile salt in the circulation when fed a 0.5% cholate diet. Therefore, it was important to investigate whether these animals hyperabsorb bile salts in general and secondary bile salts in particular, because these are particularly cytotoxic. The experiments reported here do not support the hypothesis that Atp8b1-deficient mice hyperabsorb bile salts. On a control diet the difference between portal and peripheral bile salt concentrations (as an estimate of the amount of absorbed bile salts) was the same as in control mice. Upon cholate feeding we found again that these animals accumulate very high concentrations of bile salts in their peripheral blood, but this is not associated with a significantly higher corrected portal blood concentration of bile salts in *Atp8b1*$^{G308V/G308V}$ mice versus wild-type mice (Figure 5B vs 5D, black bars). Both groups of mice have higher corrected portal blood concentrations of taurocholate upon cholate feeding in metabolic cages compared to conventional cages. The cause of this is unclear, but it might indicate that the presence of unconjugated dihydroxy bile salts (or other compounds) in the consumed feces slightly inhibits uptake of taurocholate. This higher concentration of taurocholate in portal blood, however, did not lead to higher peripheral bile salt concentrations for mice in metabolic cages as compared to conventional cages. Hence from the cholate feeding experiments in both conventional and metabolic cages we may infer that the accumulation of bile salts in the circulation of *Atp8b1*$^{G308V/G308V}$ mice is not caused by hyperabsorption of cholate or deoxycholate in the gut. Interestingly, we observed that *Atp8b1*$^{G308V/G308V}$ mice had high levels (>30 μM) of tetrahydroxy bile salt in their peripheral circulation (Figure 4C). Tetrahydroxy bile salts are normally only found in trace amounts and only reach substantial levels under cholestatic conditions in humans[15] and mice.[16] In contrast much lower concentrations were found in portal blood of *Atp8b1*$^{G308V/G308V}$ mice. This observation in *Atp8b1*$^{G308V/G308V}$ mice supports the hypothesis that impaired hepatic secretion rather than intestinal hyperabsorption is the underlying cause of the peripheral blood bile salt accumulation in the *Atp8b1*$^{G308V/G308V}$ mice. Recent studies by Paulusma et al.[17] found that bile salts have a higher retention time in the liver and are therefore more prone to rehydroxylation. These studies also demonstrated a direct impairment of hepatic transport in these mice.

In summary, we show that coprophagy in mice has little influence on the peripheral bile salt composition when these animals are on a normal diet. However, in mice fed a cholate-containing diet, coprophagy increases the portal and peripheral blood concentrations of secondary bile salts. These effects of coprophagy on the composition of the bile salt pool should be taken into account in studies involving bile salt feeding.

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