Towards novel strategies to improve lipid homeostasis - targeting the intestine
Wulp, Mariëtte Ymkje Maria van der

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Chapter 5

Genetic inactivation of the bile salt export pump in mice profoundly increases fecal cholesterol excretion

Mariëtte Y.M. van der Wulp 1,2, Theo H. van Dijk 2, Vincent W. Bloks 2, Albert K. Groen 1,2, Henkjan J. Verkade 1,2

1 Top Institute Food and Nutrition, Wageningen, The Netherlands
2 Pediatric Gastroenterology and Hepatology, Department of Pediatrics, Beatrix Children's Hospital, Groningen, University Institute for Drug Exploration, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands

In preparation
Abstract

Objectives The bile salt export pump (Bsep) is the major hepatobiliary bile salt (BS) transporter, facilitating transfer of BS from hepatocytes into the bile canaliculus. Bsep<sup>−/−</sup> mice were previously shown to escape severe cholestasis by increasing biliary BS hydrophilicity, resulting in activation of alternative transporters. Bsep<sup>−/−</sup> mice surprisingly displayed increased biliary cholesterol secretion. The consequence of Bsep deficiency and increased biliary cholesterol secretion for intestinal sterol handling is unknown. To determine these downstream effects of Bsep deficiency we determined BS and whole body cholesterol fluxes.

Methods Mice were fed either a low or a high fat diet (LFD or HFD, respectively), since it was shown previously that a HFD can induce cholesterol excretion. We determined cholesterol intake, biliary secretion, absorption and synthesis, output (feces), and the origin of fecal sterols.

Results Results were essentially the same in Bsep<sup>−/−</sup> mice fed a LFD or a HFD versus control mice. Here, only results of LFD are presented. Biliary BS secretion was decreased in Bsep<sup>−/−</sup> compared with Bsep<sup>+/+</sup> mice (-40%; p<0.01). Biliary cholate secretion was decreased (-85%; p<0.001), whereas beta-muricholate secretion was increased (+55%; p<0.01) in Bsep<sup>−/−</sup> mice. Cholesterol intake (+25%; p<0.001), biliary secretion (4 fold; p<0.001) and synthesis (11 fold; p<0.001) were increased, whereas cholesterol absorption was decreased (-90%; p<0.001) in Bsep<sup>−/−</sup> mice. Bsep<sup>−/−</sup> mice showed increased fecal cholesterol excretion (8 fold; p<0.001), mostly via the transintestinal pathway (22 fold increase; p<0.001).

Conclusion Bsep<sup>−/−</sup> mice show increased (transintestinal) cholesterol excretion, which appears to be induced by severely impaired cholesterol absorption in the presence of a hydrophilic BS pool. Potential health benefits of an increasingly hydrophilic BS pool in terms of intestinal cholesterol excretion require further research.
Introduction

Bile salts (BS) are produced by enzymatic modification of cholesterol in liver hepatocytes. They are secreted by hepatocytes into bile canaliculi, to end up with bile in the small intestine. In the small intestine, BS are indispensable for adequate absorption of lipids (dietary fat and cholesterol) and fat-soluble vitamins. In the terminal ileum, BS are efficiently reclaimed by absorption (~95%) to continue their enterohepatic circulation.\(^1\)

The main transporter facilitating transfer of BS from hepatocyte to bile canaliculus is the adenosine triphosphate-dependent binding cassette transporter B11 (ABCB11), or bile salt export pump (BSEP).\(^2,3\) Mutations in the Bsep gene can result in several forms of intrahepatic cholestasis.\(^4,5\) Heterozygous Bsep mutations have been identified in patients with intrahepatic cholestasis of pregnancy and drug-induced cholestasis.\(^5\) Missense Bsep mutations predominate in a relatively mild form of cholestatic disease termed benign recurrent intrahepatic cholestasis type 2 (BRIC-2).\(^6\) Frequently, Bsep mutations result in the absence of canalicular BSEP protein\(^7,\) and are associated with biliary BS concentrations of less than 1% of normal.\(^3\) Absence of functional BSEP results in progressive familial intrahepatic cholestasis type 2 (PFIC-2), a disease characterized by early onset of severe intrahepatic cholestasis, often requiring liver transplantation within the first decade of life.\(^3,8\)

Murine Bsep and human BSEP were previously shown to possess comparable affinities for different BS.\(^9\) Bsep mainly transports conjugated di- and tri-hydroxylated BS, such as cholate (the main BS of the human and rodent body BS pool) and chenodeoxycholate.\(^10,11\) Bsep\(^{-/-}\) mice were created to study the effects of Bsep deficiency in detail in vivo.\(^12\) An unexpected finding was that these mice displayed a preserved basal bile flow rate.\(^12-15\) Whereas the secretion of hydrophobic BS (cholate, deoxycholate and chenodeoxycholate) was severely impaired, secretion of more hydrophilic BS (ursodeoxycholate and muricholates) remained relatively unchanged.\(^12,15\) Interestingly, Bsep\(^{-/-}\) mice secreted substantial amounts of tetra-hydroxylated BS in their bile, which were not found in Bsep\(^{+/+}\) mice.\(^12,14-16\)

It was shown that the multidrug resistance 1 protein could serve as a salvage (low affinity / high capacity) pathway for BS secretion in Bsep\(^{-/-}\) mice.\(^13,15\) Bsep\(^{-/-}\) mice showed uncoupling of secretion of cholesterol and phospholipids with BS. In Bsep\(^{+/+}\), both biliary cholesterol and phospholipid secretion were significantly increased.\(^12\)

Cholesterol homeostasis is mainly regulated by its intestinal absorption, fecal excretion and de novo synthesis.\(^17\) The influence of increased biliary cholesterol secretion for its intestinal handling are unknown. The aim of the current study was to determine parameters of total body cholesterol homeostasis in Bsep\(^{-/-}\) mice and their wildtype littermates (Bsep\(^{+/+}\)). Our data show that increased hydrophilic BS secretion in Bsep\(^{-/-}\) mice is associated with severely impaired cholesterol absorption and increased fecal cholesterol output, mainly via the transintestinal pathway.
Materials and Methods

Materials

Intralipid® (20%) was obtained from Fresenius Kabi, Den Bosch, The Netherlands. 2,2,4,4,6-Deuterium-cholesterol (D₃-cholesterol) was obtained from Medical Isotopes and 25,26,26,26,27,27,27-Deuterium-cholesterol (D₇-cholesterol) from Cambridge Isotope Laboratories Inc. 1-¹³C-acetate was obtained from Sigma Aldrich (St. Louis, MO). All isotope were of 98-99% isotopic purity. Sucrose and Trizma® base were obtained from Sigma Aldrich (St. Louis, MO). Trimethylchlorosilane was obtained from Thermo Scientific, Rockford, IL. Hydrochloric acid 37%, methanol, hexane and pyridine were obtained from Merck, Darmstadt, Germany. Heptane was obtained from Rathburn chemicals ltd, Walkerburn, Scotland and N,O-bis-(trimethyl)trifluoroacetamide (BSTFA) from Supelco, Bellefonte, PA.

Mice and diet

Mice were housed in a light- and temperature-controlled facility. Tap water and food were available ad libitum. Mice were maintained on standard low fat diet (LFD) (RMH-B, 5 wt% fat, 0.66 µmol cholesterol.g⁻¹) or high fat diet (HFD) (#4141.07, 16 wt% (34 energy%) fat, 0.19 µmol cholesterol.g⁻¹), both obtained from Arie Blok BV (Woerden, The Netherlands). Bsep⁻/⁻ mice on a mixed background ¹² had been backcrossed with C57BL/6 mice for at least 10 generations. Due to the low birth rate of Bsep⁻/⁻ mice, it was inevitable to use mice of different ages. Every mouse in the Bsep⁻/⁻ group of a particular age was paired with a wildtype (Bsep⁺/+) littermate of the same age. For the cholesterol kinetic study, male Bsep⁻/⁻ and Bsep⁺/⁺ mice 4-15 months of age (LFD, n= 6 per group), and male Bsep⁻/⁻ and Bsep⁺/⁺ mice 3-5 months of age (HFD, n= 5 per group) were used. The experiments were performed in conformity with Public Health Service policy and in accordance with the national laws. The Ethics Committee for Animal Experiments of the University Medical Center of Groningen approved the experimental protocols.

Cholesterol kinetic study

The LFD was supplied to the mice from weaning until the experiment. The HFD was fed for 10 weeks before the start of the experiment. Before any intervention, baseline bloodspots were collected on filter paper from the tail vein and feces were collected during a 24h period (day -1). Food pellet weight was determined before and after the 24h feces collection period and pellets were collected for quantification of cholesterol content.

At day 1 mice received an intravenous (retro-orbital) injection of 1.5 mg D₃-cholesterol dissolved in 500 µl intralipid and an oral dose of 3 mg D₃-cholesterol dissolved in 1 ml medium chain triglyceride oil. At time points 3, 6, 12, 24, 48, 72, 96, 120, 144 and 168h (day 8) after labeled cholesterol administration, bloodspots were obtained. After taking the bloodspot at time point 168h, mice were switched to tap water containing 2% stable isotope labeled 1-¹³C-acetate for 72h. Bloodspots were collected 12, 24, 32, 48, 56 and 72h (day 11, low fat diet) and 24, 32, 48, 56, 72, 80, 96h (day 12, HFD experiment) after the start of 1-¹³C-acetate.
Body weight and food intake were determined and feces were collected daily during the entire experiment. At day 11 or 12 mice were anesthetized and the common bile duct was cannulated for bile collection during 30 minutes as previously described. Mice were sacrificed by cardiac puncture and cervical dislocation. The small intestine was divided into three equal segments, which were rinsed with phosphate-buffered saline (PBS 5 ml, low fat diet) and in addition with 5 ml of DEMI water containing protease inhibitor (HFD experiments, 1 tablet per 50 ml of DEMI). Livers and intestines per segment were snap frozen in liquid nitrogen and stored at -80°C.

**Analytical methods**

**BS and indirect cholesterol balance**

Biliary lipids were extracted and total plasma cholesterol and biliary cholesterol and BS concentrations were determined. Food pellets and fecal samples were ground and 50 mg was prepared for neutral sterol (cholesterol plus bacterial metabolites coprostanol and dehydrocholesterol) and BS analysis by gas chromatography (GC) as described previously. Indirect cholesterol balance was determined by subtraction of dietary cholesterol intake and hepatobiliary secretion from fecal output of neutral sterols (all calculated in μmol cholesterol.day⁻¹). Biliary BS hydrophobicity was calculated according to the method of Heuman.

**Fractional cholesterol absorption**

The procedure for this study, was modified for the influx of labeled cholesterol. Fractional cholesterol absorption (F(a)) was calculated as the ratio between fraction (area under the curve (AUC) of 7 days following label administration) of orally administered D₅-cholesterol and IV administered D₇-cholesterol, after correction for their administered doses: F(a)= (AUCoral / AUCIV) x (Dose IV / Dose oral).

**Cholesterol synthesis**

Fractional cholesterol synthesis was determined by mass isotopomer distribution analysis (MIDA) of the M₁ and M₃ mass isotopomers.

**Origin of fecal sterols**

In order to determine the origin of fecal sterols, we modified the method described by van der Veen et al.

**Neutral sterol content in intestinal lumen**

Before analyses, tubes containing flushed intestinal lumen contents were lyophilized for 48h. Aliquots of 10 (proximal and middle part of small intestine) to 50 (distal small intestine, cecum and colon) mg were used to determine neutral sterols by GC as described above.
Preparation of intestinal mucosa homogenates

Intestinal sections (proximal small intestine (SIP), middle segment of small intestine (SIM) and distal segment of small intestine (SID) were thawed (and kept) on ice and cut open. Mucosa was scraped off the interior with an object glass, transferred to a pre-weighed potter glass (2 ml) and the potter glass was weighed. One ml of ice-cold sucrose buffer (250 mM sucrose in 10 mM Trizma® base, pH 7.4) was added and the solution was homogenized by pottering (10 strokes). The solution was further homogenized by putting it through 1 ml syringes with needles of 20 and 26 G, respectively, 5-10 times with each needle. The homogenate was thoroughly vortexed and divided over 3 eppendorf cups (2 ml). Ten µl of homogenate was transferred to 2 10 ml glass tubes (tube 1 one for GC and tube 2 for GC-MS) analyses of cholesterol (M0-M7). Glass tubes and remaining homogenate were kept at -20°C until further analyses.

Determination of cholesterol concentration in intestinal mucosa

The glass tubes containing homogenate were thawed and internal standard (5 nmol 5α-cholestane) for neutral sterol quantification by GC was added to tube 1. Tube 2 was worked up for cholesterol label analyses (GC-MS). Lipids were extracted from all tubes as described previously. Cholesterol esters were hydrolyzed with 2 ml of a mixture containing 37% hydrochloric acid (15 ml), DEMI water (10 ml) and methanol (125 ml) (1h at 90°C). The mixture was evaporated under a stream of nitrogen at 55°C. After addition of 2 ml DEMI, lipids were extracted (2 times) by adding 3 ml of hexane, vortexing (30 seconds per tube), centrifuging (5 min at 2500 rpm) and transferring the top (hexane) layer to a new glass tube. The hexane was evaporated under a stream of nitrogen at 55°C. Unesterified cholesterol was derivatized using BSTFA/pyridine (1:1 v/v) with 1% trimethylchlorosilane at RT overnight. The mixture was evaporated under a stream of nitrogen at RT and samples were redissolved in 1 ml heptane containing 5% BSTFA (GC) or 150 µl heptane containing 5% BSTFA (GC-MS).

Statistical analysis

Using Brightstat, we calculated the significance of differences between groups (Bsep−/− and Bsep+/+ mice on a LFD and HFD) with Kruskal-Wallis rank tests (and in case this test indicated significant differences between groups) with Conover multiple comparisons tests. Data that were only generated in HFD fed Bsep+/+ and Bsep−/− mice were compared with Mann Whitney U tests. P-values <0.05 were considered statistically significant. Data represent median and interquartile range.

Results

Characteristics of Bsep−/− compared with Bsep+/+ mice

We compared characteristics of Bsep−/− and Bsep+/+ mice on both a LFD and a HFD (table 1). Mice consumed a slightly smaller amount of food per day on HFD compared with LFD (p<0.05), but Bsep−/− mice consumed more than Bsep+/+ mice on either diet (p<0.001). Bsep+/+ and Bsep−/− mice had similar body weights on LFD at the start and end of the experiment (only final body weight is shown).
Although the body weights on HFD did not significantly differ between genotypes at the start or at the end of the experiment (due to one relatively small Bsep+/+ mouse), Bsep-/- mice gained less weight on HFD compared with Bsep+/+ mice (p<0.01). Bsep-/- mice showed a higher fecal output compared with Bsep+/+ mice on either diet (p<0.001). Both Bsep+/+ and Bsep-/- mice showed increased plasma cholesterol levels on HFD compared with LFD (p<0.001). Bsep-/- mice however had significantly lower plasma cholesterol levels than Bsep+/+ mice on LFD (p<0.01) and HFD (p<0.05).

Table 1. Characteristics of Bsep-/- and Bsep+/+ mice on low and high fat diet

<table>
<thead>
<tr>
<th>Diet</th>
<th>Bsep+/+ LFD</th>
<th>Bsep+/+ HFD</th>
<th>Bsep-/- LFD</th>
<th>Bsep-/- HFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal body weight (g)</td>
<td>35.8 [28.6-36.9]</td>
<td>31.9 [29.0-34.9]</td>
<td>30.8 [23.0-32.0]</td>
<td>32.6 [31.7-34.2]</td>
</tr>
<tr>
<td>Body weight (g) at termination</td>
<td>35.8 [28.6-36.9]</td>
<td>31.9 [29.0-34.9]</td>
<td>40.9 [30.7-42.3]</td>
<td>36.8 [36.4-38.5]</td>
</tr>
<tr>
<td>Growth (g)</td>
<td>10.2 [7.5-11.2] a</td>
<td>4.4 [4.0-5.0] b</td>
<td>4.2 [4.0-5.0] a</td>
<td>4.2 [4.0-5.0] b</td>
</tr>
<tr>
<td>Bile flow</td>
<td>3.1 [2.4-3.6] a</td>
<td>4.3 [4.1-4.6] b</td>
<td>1.7 [1.3-2.5] c</td>
<td>3.6 [3.1-4.1] a</td>
</tr>
<tr>
<td>Small intestine (cm)</td>
<td>32.5 [31.0-34.0] a</td>
<td>37.0 [33.5-38.0] b</td>
<td>31.5 [29.5-34.0] c</td>
<td>33.5 [30.0-36.0] b</td>
</tr>
<tr>
<td>Colon (cm)</td>
<td>6.0 [5.5-6.5] a</td>
<td>7.0 [6.5-6.8] b</td>
<td>7.0 [6.5-7] c</td>
<td>6.5 [6.5-8] b</td>
</tr>
<tr>
<td>Cecum (g)</td>
<td>0.6 [0.4-0.6] a</td>
<td>0.6 [0.4-0.8] a</td>
<td>0.2 [0.2-0.3] b</td>
<td>0.3 [0.2-0.3] b</td>
</tr>
<tr>
<td>Food intake (g.day⁻¹)</td>
<td>4.0 [3.7-4.2] a</td>
<td>4.6 [4.5-4.9] b</td>
<td>3.5 [3.4-3.7] c</td>
<td>4.3 [4.0-4.6] d</td>
</tr>
<tr>
<td>Fecal output (g.day⁻¹)</td>
<td>0.6 [0.6-0.7] a</td>
<td>1.0 [0.9-1.2] b</td>
<td>0.4 [0.4-0.4] c</td>
<td>0.7 [0.6-0.7] a</td>
</tr>
<tr>
<td>Plasma parameters</td>
<td>Cholesterol (mmol.L⁻¹)</td>
<td>2.4 [2.1-2.7] a</td>
<td>0.7 [0.7-0.9] b</td>
<td>4.8 [3.4-5.0] c</td>
</tr>
</tbody>
</table>

Data represent median and interquartile range, n= 5-6 mice per group. Different letters indicate significant differences between groups, one or more letters in common indicate nonsignificant differences between groups.

On HFD both Bsep+/+ and Bsep-/- mice showed a lower bile flow rate than on LFD (p<0.001), but Bsep-/- mice displayed a significantly higher flow rate than Bsep+/+ mice (p<0.01). Biliary BS secretion was 40% lower in Bsep-/- compared with Bsep+/+ mice on LFD (p<0.001; figure 1A), decreased in both groups on HFD (p<0.01) and was comparable in Bsep-/- and Bsep+/+ mice on HFD. On either diet, Bsep-/- mice showed a decreased biliary secretion of cholate (the main BS in Bsep+/+ mice) compared with Bsep+/+ mice (LFD -87%; p<0.001; HFD -82%; p<0.001; figure 1B). On the other hand Bsep-/- mice showed increased biliary secretion of β-muricholate (BMC, the main BS in Bsep-/- mice) compared with Bsep+/+ mice (LFD +58%; p<0.001; HFD +100%; p<0.01; figure 1C). In accordance with these data, Bsep-/- mice secreted a more hydrophilic bile in general as shown by decreased BS hydrophobicity. (Heuman index on LFD [-0.66 to -0.62] vs. -0.29 [-0.32 to -0.25]; p<0.001 and on HFD [-0.69 to -0.67] vs. -0.36 [-0.42 to -0.24]; p<0.001; data not shown).
Figure 1. Biliary bile salt secretion rate in Bsep−/− and Bsep+/+ mice on LFD (white boxes) and HFD (grey boxes). A) Total biliary bile salt secretion rate (BSSR). B) Biliary cholate secretion rate (CA SR). C) Biliary beta-muricholate secretion rate (βMC SR). Data are represented as median and interquartile range, n= 5-6 mice per group. **p<0.01, ***p<0.001 represent significant differences between the indicated two groups.

Figure 2. Fecal bile salt excretion in Bsep−/− and Bsep+/+ mice on LFD (white boxes) and HFD (grey boxes). A) Total daily fecal bile salt excretion. B) Daily fecal cholate excretion. C) Daily β-muricholate excretion. Data are represented as median and interquartile range, n=4-7 mice per group. *p<0.05, **p<0.01, ***p<0.001 represent significant differences between the indicated two groups.
Bsep−/− mice excreted less fecal BS compared with Bsep+/+ mice on LFD (-80%; p<0.001) and on HFD (-84%; p<0.001; figure 2A). On both diets, Bsep−/− excreted less cholate in their feces compared with Bsep+/+ mice (LFD -86%; p<0.01; HFD -79%; p<0.05; figure 2B). On the other hand, Bsep−/− excreted a similar amount of β-muricholate compared with Bsep+/+ mice on LFD and HFD; figure 2C).

**Bsep−/− mice display increased non-hepatobiliary cholesterol excretion (indirect cholesterol balance)**

Compared with a LFD, a HFD may induce TICE. In order to investigate the effect of Bsep deficiency on cholesterol homeostasis, we fed mice both a LFD or a HFD for 10 weeks. Attributable to the lower cholesterol content of the HFD, both Bsep+/+ and Bsep−/− mice consumed less cholesterol per day on HFD compared with LFD (p<0.001). Bsep−/− mice consumed 25% (LFD; p<0.001) to 35% (HFD; p<0.001) more cholesterol than Bsep+/+ mice (table 2). Both Bsep+/+ and Bsep−/− mice secreted less cholesterol in bile on HFD compared with LFD (p<0.001). In Bsep−/− mice biliary cholesterol secretion was increased ~4-6 fold on both diets compared with Bsep+/+ mice (table 2; p<0.001). The difference in biliary cholesterol secretion could however not account for the ~8 fold (LFD; p<0.001) and ~15 fold (HFD; p<0.001) increase in fecal cholesterol excretion that Bsep−/− displayed compared with Bsep+/+ mice. Altogether, the data indicate that Bsep−/− mice excreted a higher amount of cholesterol in feces via a non-hepatobiliary pathway than Bsep+/+ mice (table 2; p<0.001).

**Table 2. Indirect cholesterol balance**

<table>
<thead>
<tr>
<th>µmol.day⁻¹</th>
<th>Bsep+/+</th>
<th>Bsep−/−</th>
<th>Bsep+/+</th>
<th>Bsep−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LFD</td>
<td>HFD</td>
<td>LFD</td>
<td>HFD</td>
</tr>
<tr>
<td>Dietary cholesterol intake</td>
<td>2.2 [2.2-2.4] a</td>
<td>2.8 [2.5-3.1] b</td>
<td>0.5 [0.5-0.6] c</td>
<td>0.7 [0.7-0.8] d</td>
</tr>
<tr>
<td>Biliary cholesterol secretion</td>
<td>2.5 [1.8-3.3] a</td>
<td>10.2 [9.0-10.6] b</td>
<td>0.6 [0.4-0.8] c</td>
<td>3.6 [3.2-4.3] d</td>
</tr>
<tr>
<td>Fecal neutral sterol excretion</td>
<td>4.4 [3.5-5.8] a</td>
<td>34.6 [30.9-42.4] b</td>
<td>1.8 [1.8-3.2] c</td>
<td>27.9 [27.3-34.9] b</td>
</tr>
<tr>
<td>Net non-hepatobiliary cholesterol excretion (&lt;0 = net absorption; &gt;0 = net excretion)</td>
<td>-0.5 [-1.3-1.2] a</td>
<td>21.4 [19.0-29.1] b</td>
<td>0.9 [0.7-1.8] a</td>
<td>22.9 [22.7-31.0] b</td>
</tr>
</tbody>
</table>

Data represent median and interquartile range, n= 5-6 mice per group. Different letters indicate significant differences between groups (p<0.05).

**Cholesterol absorption is impaired and synthesis is upregulated in Bsep−/− mice**

We measured crucial parameters of cholesterol homeostasis, cholesterol absorption and synthesis, with stable isotope methodology. Bsep−/− mice absorbed cholesterol to a much lower extent than Bsep+/+ mice on a LFD (5 versus 43% (median); p<0.001; figure 3). On HFD, both Bsep−/− and Bsep+/+ mice absorbed more cholesterol than on LFD (Bsep+/+ LFD vs. HFD p<0.05 and Bsep−/− mice LFD vs. HFD p<0.01), but Bsep−/− mice again absorbed much less cholesterol than Bsep+/+ mice (18 versus 84% (median); p<0.001; figure 3).
Bsep\textsuperscript{-/-} mice synthesized more cholesterol compared with Bsep\textsuperscript{+/+} mice on both diets (LFD 13 fold; p<0.001 and HFD 8 fold; p<0.001; figure 4A). In both Bsep\textsuperscript{-/-} and Bsep\textsuperscript{+/+} mice, whole body cholesterol synthesis was higher on HFD compared with LFD (Bsep\textsuperscript{+/+} LFD vs. HFD p<0.001 and Bsep\textsuperscript{-/-} mice LFD vs. HFD p<0.001). Bsep\textsuperscript{-/-} mice showed a higher fraction of newly synthesized cholesterol in bile and feces (both diets, table 3), and intestinal mucosa and intestinal lumen contents (measured on HFD, table 3). Similarly, compared with Bsep\textsuperscript{+/+} mice, Bsep\textsuperscript{-/-} mice had an increased amount of (preformed and) \textit{de novo} synthesized cholesterol in bile (all p<0.001; figure 4B) and feces (all p<0.001; figure 4C), and intestine luminal contents (all p<0.05); figure 4D, measured on HFD). The amount of \textit{de novo} synthesized cholesterol in feces and bile was very small in Bsep\textsuperscript{+/+} mice. However, both Bsep\textsuperscript{-/-} and Bsep\textsuperscript{+/+} mice excreted lower amounts of \textit{de novo} synthesized fecal (p<0.05) and biliary (p<0.001) cholesterol on HFD compared with LFD (figure 4B and 4C). Bsep\textsuperscript{+/+} and Bsep\textsuperscript{-/-} mice showed similar cholesterol concentrations in small intestinal mucosa (figure 4E).

**Table 3.** Fractional cholesterol synthesis

<table>
<thead>
<tr>
<th>%</th>
<th>Bsep\textsuperscript{+/+}</th>
<th>Bsep\textsuperscript{-/-}</th>
<th>Bsep\textsuperscript{+/+}</th>
<th>Bsep\textsuperscript{-/-}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LFD</td>
<td>HFD</td>
<td>LFD</td>
<td>HFD</td>
</tr>
<tr>
<td>Bile</td>
<td>4.8 [3.6-6.1] \textsuperscript{a}</td>
<td>26.6 [25.6-27.8] \textsuperscript{b}</td>
<td>7.1 [5.9-8.7] \textsuperscript{c}</td>
<td>23.2 [19.0-25.1] \textsuperscript{d}</td>
</tr>
<tr>
<td>Feces</td>
<td>6.4 [6.1-6.9] \textsuperscript{a}</td>
<td>21.5 [20.1-22.1] \textsuperscript{b}</td>
<td>5.1 [5.1-7.7] \textsuperscript{a}</td>
<td>12.5 [11.1-15.4] \textsuperscript{c}</td>
</tr>
<tr>
<td>SIP mucosa</td>
<td>8.8 [7.6-10.7] \textsuperscript{a}</td>
<td>27.4 [21.9-28.4] \textsuperscript{b}</td>
<td>8.6 [7.5-10.8] \textsuperscript{a}</td>
<td>23.3 [17.8-24.2] \textsuperscript{b}</td>
</tr>
<tr>
<td>SIM mucosa</td>
<td>10.3 [8.4-12.2] \textsuperscript{a}</td>
<td>23.2 [17.6-23.7] \textsuperscript{b}</td>
<td>8.6 [7.5-10.8] \textsuperscript{a}</td>
<td>23.3 [17.8-24.2] \textsuperscript{b}</td>
</tr>
<tr>
<td>SID mucosa</td>
<td>8.7 [7.6-9.7] \textsuperscript{a}</td>
<td>23.6 [19.4-25.5] \textsuperscript{b}</td>
<td>10.3 [8.4-12.2] \textsuperscript{a}</td>
<td>23.2 [17.6-23.7] \textsuperscript{b}</td>
</tr>
<tr>
<td>SIP lumen contents</td>
<td>8.2 [9.2-9.6] \textsuperscript{a}</td>
<td>22.7 [18.2-24.2] \textsuperscript{b}</td>
<td>9.3 [8.2-11.2] \textsuperscript{a}</td>
<td>23.1 [17.5-23.8] \textsuperscript{b}</td>
</tr>
<tr>
<td>SIM lumen contents</td>
<td>10.6 [8.9-11.4] \textsuperscript{a}</td>
<td>22.2 [17.3-22.9] \textsuperscript{b}</td>
<td>9.9 [8.6-10.6] \textsuperscript{a}</td>
<td>21.9 [17.2-23.4] \textsuperscript{b}</td>
</tr>
<tr>
<td>Cecum lumen contents</td>
<td>10.6 [8.9-11.4] \textsuperscript{a}</td>
<td>22.2 [17.3-22.9] \textsuperscript{b}</td>
<td>9.9 [8.6-10.6] \textsuperscript{a}</td>
<td>21.9 [17.2-23.4] \textsuperscript{b}</td>
</tr>
</tbody>
</table>

Data represent median and interquartile range, n= 4-6 mice per group. Different letters indicate significant differences (p <0.05) between groups.
However, Bsep<sup>−/−</sup> mice displayed increased small intestinal length (table 1) and mucosal weight (data not shown), which resulted in an increased total amount of cholesterol in small intestinal mucosa compared with Bsep<sup>+/+</sup> mice, in which preformed and <i>de novo</i> synthesized cholesterol were increased (figure 4F).

![Figure 4](image_url)

**Figure 4.** Cholesterol synthesis in Bsep<sup>−/−</sup> and Bsep<sup>+/+</sup> mice. A) Total body synthesis on LFD (white boxes) and HFD (grey boxes). B) Preformed (black) and <i>de novo</i> synthesized (white) cholesterol in bile. C) Preformed (black) and <i>de novo</i> synthesized (white) neutral sterols in feces. D) Cholesterol concentration in different parts of the small intestine on HFD in Bsep<sup>+/+</sup> (white) and Bsep<sup>−/−</sup> (grey) mice. E) Preformed (black) and <i>de novo</i> synthesized (white) cholesterol in small intestinal mucosa on HFD. F) Preformed (black) and <i>de novo</i> synthesized (white) neutral sterol content in intestine lumenal contents of Bsep<sup>−/−</sup> and Bsep<sup>+/+</sup> mice on HFD. SIP= small intestine proximal segment, SIM= SI middle segment, SID= SI distal segment, Ce= cecum, Co= colon. Data are represented as medians (and interquartile range in E), n= 5-6 mice per group. *p<0.05, **p<0.01, ***p<0.001 represent significant differences between the indicated two groups. For significance of differences shown in figure B, C and D, see text.
Transintestinal cholesterol excretion is induced in Bsep$^{-/-}$ mice

We used the data obtained during our stable isotope studies to model the net contribution of dietary, biliary, and intestinal cholesterol to fecal sterols. 20

Bsep$^{-/-}$ mice excreted more dietary cholesterol compared with Bsep$^{+/+}$ mice on either diet (LFD 2.2 fold; p<0.001, HFD 4.5 fold; p<0.001; figure 5), due to decreased absorption as described above. Similarly, Bsep$^{-/-}$ mice excreted more biliary cholesterol on both diets (LFD 4 fold; p<0.001, HFD 29 fold; p<0.001; figure 5). Finally, Bsep$^{-/-}$ mice showed increased TICE (LFD 22 fold; p<0.001, HFD 14 fold; p<0.001; figure 5).

Discussion

The major finding in our study is that Bsep$^{-/-}$ mice, that secrete more hydrophilic BS via bile, display severely impaired cholesterol absorption, increased cholesterol synthesis and greatly induced (transintestinal) cholesterol excretion either on a low or high fat diet.

Previous studies have shown that dietary hydrophilic BS (including β-muricholate) inhibit cholesterol absorption. 25-27 In hamsters, it was shown that enrichment of bile with very hydrophilic 6-alpha-hydroxylated BS induced a global hypocholesterolemic effect and enhanced 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (Hmgcr, the rate-limiting enzyme for cholesterol synthesis) activity and fecal cholesterol excretion. 26 Cholesterol input into the intestine of Bsep$^{-/-}$ mice is increased, based on slightly more food ingestion and on a higher biliary cholesterol secretion rate. However, the severely impaired intestinal capacity to absorb cholesterol only explains ~10-40% of the amount of cholesterol lost via the feces in Bsep$^{-/-}$ mice. Apparently, in Bsep$^{-/-}$ mice a non-dietary and non-biliary source of cholesterol contributes to fecal cholesterol loss. Our data indicate that transintestinal cholesterol excretion is responsible for ~60-90% of fecal cholesterol excretion in Bsep$^{-/-}$ mice. The induction of TICE in the present model occurs together with the more hydrophilic biliary BS composition. Future studies would have to be performed to determine whether the coincidence is "merely" an association or indeed causally related.
If the latter were the case, then strategies to enhance the hydrophilicity of the BS composition could become of preventive and therapeutic potential for hypercholesterolemia. Increased biliary cholesterol secretion does not seem to facilitate gallstone formation in the presence of a very hydrophilic BS pool, which would otherwise be a drawback.

Overall, the differences between Bsep+/+ and Bsep-/- mice were highly consistent and mostly independent of the diet. Bsep-/- mice displayed increased small intestinal length and mucosal weight, possibly as a compensatory mechanism for the impaired absorption of cholesterol, and likely other nutrients. Our previous studies showed that absorption of dietary fatty acids is decreased in Bsep-/- mice (~85%) compared with Bsep+/+ mice (~95%, unpublished data). Another indicator of malabsorption is the increased fecal output we found in Bsep-/- mice.

Bsep-/- showed a decrease in plasma cholesterol compared with Bsep+/+ mice on both diets, which is likely due to several factors, including profoundly increased biliary cholesterol secretion, cholesterol malabsorption and ineffective compensatory increased cholesterol synthesis. Cohen-Solal previously showed increased biliary cholesterol secretion upon oral hydrophilic BS feeding, however, Wang et al. in contrast showed the opposite. In the presence of low intracellular cholesterol levels, activity of the transcription factors sterol regulatory element-binding proteins (SREBPs) is increased and genes involved in cholesterol synthesis (Hmgcr) and uptake (Low density lipoprotein receptor (Ldlr)) are induced. Although hepatic Ldlr mRNA expression in Bsep-/- mice is not evidently increased, its activity is unknown. On the other hand, the nuclear receptor Liver X receptor (Lxr) is a major transcriptional regulator of cholesterol homeostasis, particularly in terms of cholesterol disposal from the body. Lxr activated transcription of the two half-transporters ATP-binding cassette sub-family G member 5 and 8 (Abcg5/g8), which facilitate cholesterol transfer from liver to bile and from enterocyte to intestinal lumen, may play a role in Bsep-/- mice as well. Future studies are warranted to provide more insight into the mechanism behind the profoundly decreased plasma cholesterol, increased biliary cholesterol secretion, and increased TICE in Bsep-/- mice.

Altogether, our data clearly show that absence of Bsep in the mouse liver is associated with secretion of hydrophilic BS and profound transintestinal cholesterol excretion. This provides a rationale for studying the effect of hydrophilic BS feeding to hypercholesterolemic mice and perhaps humans on fecal disposal of cholesterol.

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