Supporting Materials and Methods

Determination of biliary lipid output. To perform biliary lipid secretion study, the common bile duct was cannulated with a PE-10 polyethylene catheter (1). After successful catheterization and flow of fistula bile, cholecystectomy was performed. The first hour sample of hepatic bile was collected by gravity in mice (n=5 per group) and bile volume was determined. All bile samples were frozen and stored at –20°C for further lipid analyses.

Because plasma HDL, rather than VLDL or LDL, preferentially provides sterols to the liver for secretion into bile (2), the effect of the ABCG5/G8-independent pathway on hepatic secretion of biliary sterols was investigated further. Exactly 20 mL of plasma from WT mice was incubated with a Whatman filter paper impregnated with [^{3}H]sitostanol and [^{14}C]cholesterol at 4°C for 12 hours. The HDL ($1.050 \leq d \leq 1.210$ g/mL) fraction was isolated in a Beckman Model L8-80M preparative ultracentrifuge using a type SW41 Ti rotor at 4°C and 40,000 rpm for 24 hours. The radiolabeled HDL was dialyzed overnight against 0.9% NaCl and 0.01% EDTA at pH 7.4, and then passed through a 0.22 μm Millipore filter to remove particulate cholesterol and proteins. To determine the transport rate of [^{3}H]sitostanol and [^{14}C]cholesterol in HDL from plasma into bile, i.e., the reverse cholesterol transport pathway, the common bile duct was cannulated according to previously described methods (1). Following cholecystectomy, as well as successful catheterization and flow of fistula bile, identical amounts of [^{3}H]sitostanol- and [^{14}C]cholesterol-labeled HDL in a volume of 100 μL were injected via the jugular vein as a bolus to chow-fed WT, ABCG5(-/-)/G8(-/-) and ABCG8 (-/-) mice

(n=5 per group). Immediately after injection, hepatic bile samples were collected by gravity for 6 hours and total radioactivity in these hepatic bile samples was counted.

Biliary lipid analyses. The measurement of biliary cholesterol, phospholipid, and total bile salt concentrations, as well as individual bile salt species has been described elsewhere (3). Cholesterol saturation index (CSI) and hydrophobicity index of bile samples were calculated from critical tables (4) and according to Heuman's method (5), respectively. Relative lipid composition of pooled gallbladder bile and individual hepatic bile was plotted on condensed phase diagrams, in which the phase boundaries of the micellar zone and the crystallization pathways were extrapolated from model bile systems developed for taurocholate at 37°C and at a total lipid concentration of 10 g/dL for pooled gallbladder bile and of 3 g/dL for individual hepatic bile (6).

Gallbladder contraction function. To examine the effect of the ABCG5/G8-independent pathway on gallbladder contractility, a dynamic measurement of gallbladder emptying function was carried out in mice (n=5 per group) in response to a high-fat meal or to exogenously administered sulfated cholecystokinin octapeptide (CCK-8) after 14 days on the lithogenic diet as described elsewhere (7).

Quantitative real-time PCR assay. Total RNA was extracted from mouse liver and gallbladder tissues (n=4 per group) using RNeasy Midi (Qiagen, Valencia, CA). Reverse-transcription reaction was performed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA) with 1 μg of total RNA and random hexamers to generate cDNA. The sequences of the primers and probes for *Abcg5*, *Abcg8*, acyl-CoA:cholesterol acyltransferase, isoform 2 (*Acat2*), cholecystokinin-1 receptor (*Cck-1r*), cholesterol 7α-hydroxylase (*Cyp7a1*), sterol 27-hydroxylase

(*Cyp27a1*), Niemann-Pick C1-like 1 (*Npc1l1*), scavenger receptor class B, member 1 (*Sr-b1*), and *β-Actin* have been described elsewhere (7).

Western blot analysis. Whole-liver extracts and anti-ABCG5 and anti-ABCG8 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used for immunoblot analysis according to published methods (8). Equivalent loading of lanes was verified by reprobing blots with mouse antibody against β-Actin (Sigma, St. Louis, MO). All blots were incubated with horseradish-peroxidase-conjugated sheep anti-rabbit IgG, and then were subjected to enhanced chemiluminescence detection (Amersham, Arlington Heights, IL). Each experiment was performed in triplicate to ensure reproducibility. Protein quantification was performed by densitometric analysis of films using the NIH ImageJ software.

Statistical methods. Values are presented as mean ± SD. Data were analyzed using Student's *t*-test, Mann-Whitney U-test, or Chi-square test, as appropriate. If the F-value was significant, comparisons among groups of mice were further analyzed by a multiple *post hoc* comparison test. Analyses were performed with the *SuperANOVA* software (Abacus Concepts, Berkeley, CA). A two-tailed probability of <0.05 was considered statistically significant.

Supporting Results

Supplementary Table S1 lists the relative biliary lipid composition of pooled gallbladder bile in WT, ABCG5(-/-)/G8(-/-), and ABCG8 (-/-) mice before (day 0, on chow) and during feeding the lithogenic diet for 56 days.

Supplementary Table S2 lists relative biliary lipid composition of individual hepatic bile in WT, ABCG5(-/-)/G8(-/-), and ABCG8 (-/-) mice after 56 day of the lithogenic diet feeding.

Supporting References:

1. Wang DQ, Lammert F, Paigen B, Carey MC. Phenotypic characterization of *Lith* genes that determine susceptibility to cholesterol cholelithiasis in inbred mice: pathophysiology of biliary lipid secretion. J Lipid Res 1999;40:2066-2079.
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4. Carey MC. Critical tables for calculating the cholesterol saturation of native bile. J Lipid Res 1978;19:945-955.
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7. de Bari O, Wang HH, Portincasa P, Liu M, Wang DQ. The deletion of the estrogen receptor alpha gene reduces susceptibility to estrogen-induced cholesterol cholelithiasis in female mice. Biochim Biophys Acta 2015;1852:2161-2169.
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Supplementary Table S1. Biliary lipid composition of gallbladder bile during cholesterol crystallization and gallstone formation

Day	Mole% Ch	Mole% PL	Mole% BS	Ch/PL	Ch/BS	[TL] (g/dL)	CSI
Wild-type							
0	3.88	11.96	84.17	0.325	0.046	7.08	0.89
14	6.88	16.40	76.72	0.419	0.090	9.01	1.19
28	8.20	16.14	75.67	0.508	0.108	8.94	1.42
56	9.02	16.62	74.36	0.542	0.121	9.13	1.52
ABCG5(-/-)/G8(-/-)							
0	1.61	11.70	86.69	0.137	0.019	6.53	0.38
14	5.67	15.77	78.56	0.359	0.072	8.26	1.03
28	6.10	16.23	77.68	0.376	0.078	8.54	1.08
56	6.51	16.86	76.63	0.386	0.085	8.66	1.12
ABCG8 (-/-)							
0	1.74	12.12	86.14	0.144	0.020	6.76	0.40
14	5.56	15.20	79.24	0.366	0.070	8.34	1.04
28	6.34	17.26	76.40	0.368	0.083	8.42	1.08
56	6.91	16.64	76.45	0.415	0.090	8.46	1.20

Values were measured from pooled gallbladder bile (n=5 per group). Abbreviations: Ch, cholesterol; PL, phospholipids; BS, bile salts; [TL], total lipid concentration; CSI, cholesterol saturation index.

Supplementary Table S2. Biliary lipid composition of individual hepatic bile after 56 days of the lithogenic diet feeding

Mice	Mole% Ch	Mole% PL	Mole% BS	Ch/PL	Ch/BS	[TL] (g/dL)	CSI
Wild-type							
1	8.90	18.75	72.35	0.475	0.123	2.765	1.73
2	8.05	17.35	74.59	0.464	0.108	3.163	1.62
3	7.92	15.80	76.27	0.502	0.104	3.229	1.70
4	7.62	17.01	75.37	0.448	0.101	3.223	1.56
5	9.58	19.35	71.07	0.495	0.135	2.986	1.79
Mean±SD	8.42±0.81	17.65±1.41	73.93±2.16	0.48±0.02	0.11±0.01	3.07±0.20	1.68±0.09
ABCG5(-/-)/G8(-/-)							
1	5.31	15.09	79.61	0.352	0.067	2.339	1.30
2	8.14	19.85	72.01	0.410	0.113	2.378	1.58
3	5.99	15.14	78.87	0.396	0.076	2.652	1.41
4	5.95	14.14	79.91	0.420	0.074	2.543	1.48
5	4.95	16.47	78.59	0.300	0.063	2.821	1.09
Mean±SD	6.06±1.24*	16.14±2.23	77.80±3.28	0.38±0.05*	0.08±0.02$^{\#}$	2.55±0.20*	1.37±0.19$^{\#}$
ABCG8 (-/-)							
1	6.74	20.11	73.15	0.335	0.092	2.319	1.33
2	6.17	14.75	79.08	0.419	0.078	2.678	1.47
3	5.59	15.42	79.00	0.362	0.071	2.594	1.31
4	5.67	12.65	81.68	0.448	0.069	2.776	1.49
5	4.79	17.80	77.41	0.269	0.062	2.529	1.02
Mean±SD	5.79±0.73*	16.14±2.88	78.06±3.15	0.37±0.07$^{\#}$	0.07±0.01*	2.58±0.17*	1.32±0.19*

Abbreviations: Ch, cholesterol; PL, phospholipids; BS, bile salts; [TL], total lipid concentration; CSI, cholesterol saturation index.
Compared to wild-type mice, $^{\#}$P<0.05; *P<0.01; and **P<0.001.