Hepatic Expression of ABC Transporters G5 and G8 Does Not Correlate With Biliary Cholesterol Secretion in Liver Transplant Patients

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The adenosine triphosphate (ATP)-binding cassette (ABC)-transporters ABCG5 and ABCG8 have been shown to mediate hepatic and intestinal excretion of cholesterol. In various (genetically modified) murine models, a strong relationship was found between hepatic expression of ABCG5/ABCG8 and biliary cholesterol content. Our study aimed to relate levels of hepatic expression of ABCG5 and ABCG8 to biliary excretion of cholesterol in man. From 24 patients who had received a liver transplant, bile samples were collected daily after transplantation over a 2-week period to determine biliary composition. Expression of ABCG5, ABCG8, MDR3, and BSEP was assessed by real-time polymerase chain reaction (PCR) in liver biopsy specimens collected before and after transplantation. Levels of hepatic ABCG5, ABCG8, and MDR3 messenger RNA (mRNA) were strongly correlated. After transplantation, the biliary secretion rate of cholesterol continuously increased, coinciding with gradual increases in bile salt and phospholipid secretion. In contrast, hepatic levels of ABCG5 and ABCG8 mRNA remained unchanged. Surprisingly, no correlation was found between the hepatic expression of ABCG5 and ABCG8 and rates of biliary cholesterol secretion, normalized for biliary phospholipid secretion. As expected, the concentration of biliary phospholipids correlated well with MDR3 expression. In conclusion, the strong relationship between ABCG5 and ABCG8 gene expression is consistent with the coordinate regulation of both genes, and in line with heterodimerization of both proteins into a functional transporter. Hepatic ABCG5/ABCG8 expression, at least during the early phase after transplantation, is not directly related to biliary cholesterol secretion in humans. This finding suggests the existence of alternative pathways for the hepatobiliary transport of cholesterol that are not controlled by ABCG5/ABCG8. (HEPATOLOGY 2005;42:1166-1174.)

Cholesterol is critical in human metabolism. It serves as a precursor of steroid hormones and bile salts and as a fundamental structural constituent of (intra) cellular membranes. The intestine and liver play important roles in the maintenance of cholesterol homeostasis.1 Cholesterol is acquired through intestinal absorption from dietary sources and through endogenous synthesis from acetyl-CoA, which mainly occurs in the liver.2 In the liver, cholesterol is redistributed via circulation after its incorporation into very-low-density lipoproteins, or secreted into the bile after conversion into bile salts or as free cholesterol.2 The molecular mechanisms that are involved in the hepatobiliary secretion of cholesterol have been studied extensively.3,4 Recently, it was shown that adenosine triphosphate (ATP)-binding cassette (ABC) transporters, such as ABCG5 and ABCG8, are strongly involved in this process.5,6 The genes encoding these proteins are highly expressed in the liver and intestine of mice and humans.7,8 Mutations in either of the two genes encoding these ABC transporters underlie the autosomal recessive disease sitosterolemia.7,9,10 Affected subjects show strongly increased intestinal absorption of dietary plant sterols (e.g., sitosterol) and of cholesterol, and impaired biliary sterol output. This disorder is characterized by markedly elevated plasma plant sterol con-
centrations, development of premature atherosclerosis, and depositions of sterols in tendons and skin. A large body of evidence indicates that ABCG5 and ABCG8 are half-transporters that dimerize to generate a functional protein. In a polarized rat hepatoma cell line, Graf et al. showed that ABCG5 and ABCG8 co-localize at the canalicular membrane and presented evidence that both dimerization partners are required for their trafficking to the apical cell surface. Disruption of both genes, simultaneously or individually, in mice leads to extremely low concentrations of biliary cholesterol. Conversely, pharmacological induction of endogenous murine Abcg5 and Abcg8 and overexpression of human ABCG5 and ABCG8 in transgenic mice significantly promotes hepatobiliary excretion of cholesterol. Transcription of Abcg5 and Abcg8 is induced on activation of the nuclear hormone receptor liver X receptor (LXR). Treatment of wild-type mice with an LXR agonist increased Abcg5/g8 expression and secretion of cholesterol into bile, and LXR knockout mice fail to increase Abcg5/g8 expression in response to cholesterol feeding. Kosters et al. found a strong linear correlation between levels of hepatic Abcg5 and Abcg8 gene expression and biliary cholesterol secretion across a number of (genetically modified) murine models with either decreased or induced output rates of biliary cholesterol. In heterozygous (Abcg5+/−/Abcg8+/−) mice, levels of biliary cholesterol were approximately 50% of those in wild-type animals. Likewise, biliary cholesterol content was shown to be reduced in Abcg5+/− and Abcg8+/− only mice. In mice expressing up to 16 copy numbers of both genes, a significant positive linear relationship between levels of Abcg5/Abcg8 messenger RNA (mRNA) and concentrations of biliary cholesterol was also observed. Reduced Abcg5 and Abcg8 expression in livers of streptozotocin-diabetic rats was associated with reduced biliary cholesterol content. Together, these studies established strong relationships between levels of hepatic Abcg5/Abcg8 mRNA and the biliary output of cholesterol in rodents, indicative for a high degree of control exerted by the transporter pair.

We examined the relationship between the expression of hepatic ABCG5 and ABCG8 and the biliary secretion of cholesterol in patients who underwent successful orthotopic liver transplantation (OLT). Surprisingly, our data demonstrate that, in this specific population of patients, no direct relationship exists between the expression of ABCG5/ABCG8 transporter genes and the biliary output of cholesterol.

Patients and Methods

Patients and Tissue Specimens. Specimens of liver tissue were obtained during routine diagnostic biopsies from 24 grafts. According to the Groningen liver transplant protocol, three consecutive needle biopsies were collected: at the end of cold preservation, approximately 3 hours after reperfusion, and 1 week after transplantation. An aliquot of the biopsy specimen was immediately snap-frozen in liquid nitrogen for isolation of total RNA. The remaining material was used for routine histological analysis. Before transplantation, the gallbladder was removed from the donor liver. During the transplantation, after revascularization of the graft, a catheter was inserted in the common bile duct in accordance with our protocol. Via this open biliary tube, bile flow was entirely diverted outside the patient into a collection bag that was placed below the horizontal bed level. Interruption of the enterohepatic circulation in the patient was prevented through readministration of bile in the small intestine via a percutaneous feeding jejunostomy catheter. Samples of bile were collected daily in the first postoperative week, and were collected 3 times in the second week. Bile was collected between 8:00 and 9:00 AM for measurements of total bile salt, phospholipid, and cholesterol concentrations. Bile volume was assessed gravimetrically. Postoperative immunosuppression was based on tacrolimus or cyclosporin and a rapid taper of steroids. Donor and recipient demographics and surgical variables are listed in Table 1. Normal liver tissue (n = 5) was collected from resections for tumors. Patients had given written informed consent before sampling of tissue specimens. All liver biopsies and bile samples were stored at −80°C.

Bile Analysis. Bile was analyzed for total bile salt, phospholipid, and cholesterol contents. Total bile salt concentrations were measured spectrophotometrically with 3α-hydroxysteroid dehydrogenase. Bile salt composition was analyzed by capillary gas chromatography as described earlier. The hydrophobicity index of individual bile salts was calculated using indices described by Heman et al. and using estimated 3:1 glycine/taurine bile salt conjugation ratio. Phospholipid concentrations in bile were assayed using a commercially available enzymatic method (Wako Chemicals GmbH, Neuss, Germany). Biliary cholesterol concentrations were determined enzymatically with cholesterol oxidase (Roche Diagnostics GmbH, Mannheim, Germany). Postoperatively, total bile volume was measured daily to calculate bile flow. Bile flow was expressed as daily bile production per kilogram body weight of the donor.

RNA Extraction and Reverse Transcription Polymerase Chain Reaction. Isolation of total RNA was per-
Formation of bile salt export pump (BSEP; gene symbol ABCB11), resistance protein 3 (MDR3; gene symbol ABCB4), the scavenger receptor class B type I (SR-BI) were measured by real-time PCR. Messenger RNA levels of ABCG5 and ABCG8 and the scavenger receptor class B type I (SR-BI) were measured by real-time PCR (Fig. 1A). Biliary bile salt secretion, known to be the main driving force for bile production, increased curvilinearly (Fig. 1B). Concomitantly, the biliary excretion rates of phospholipids and cholesterol significantly increased during the first postoperative week (Fig. 1C-D) and continued to rise during the second week.

When output rates of bile salts, phospholipids, and cholesterol were plotted against each other, strong positive correlations were found (Fig. 2A-C), as has been previously reported.29 When bile salt output approached a zero secretion rate, no phospholipid or cholesterol secretion was observed (Fig. 2A-B). Thus, it appears that hepatobiliary lipid secretion proceeds normally under posttransplantation conditions.

**Hepatic ABCG5 and ABCG8 Expression Does Not Correlate With Biliary Cholesterol Secretion in Patients After Liver Transplantation.** By using real-time PCR, levels of transporter mRNA were analyzed in control livers and in liver grafts before transplantation, 3 hours after resuming blood circulation of the transplant, and 1 week after the operation. Messenger RNA copy numbers of transporter genes were normalized to those of 18S rRNA.

Liver biopsies collected 1 week after transplantation were used to compare levels of ABCG5 and ABCG8 transporter expression with the biliary secretion of cholesterol. At this time, biliary lipid secretion was analyzed in detail.

### Table 1. Demographic Data of Donors and Recipients and Surgical Variables (n = 24)

<table>
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<tr>
<th>Donor variables</th>
<th>Surgical Variables (n = 24)</th>
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<tbody>
<tr>
<td>Age, years; mean (range)</td>
<td>Cold ischemia time, CIT (minutes; mean ± SEM) 522 (±27)</td>
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<tr>
<td>Sex (M/F)</td>
<td>Warm ischemia time, WIT (minutes; mean ± SEM) 45 (±2)</td>
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<td>Causes of death, number (%)</td>
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<td>Intracerebral bleeding or infarction</td>
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<td>Trauma</td>
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<td>Meningitis</td>
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<td>Other</td>
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<td>Age, years; mean (range)</td>
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<td>Gender (M/F)</td>
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<td>Disease, number (%)</td>
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<td>Postnecrotic cirrhosis</td>
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<td>Hepatitis C virus</td>
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<td>Hepatitis B virus</td>
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<td>Autoimmune hepatitis</td>
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<td>Liver cirrhosis n.o.s.</td>
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<td>Alcoholic cirrhosis</td>
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<td>Biliary cirrhosis</td>
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<td>Primary sclerosing cholangitis</td>
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<td>Primary biliary cirrhosis</td>
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<td>Biliary cirrhosis</td>
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<td>Metabolic disorder</td>
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<td>Familial amyloid polyneuropathy</td>
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<td>M. Wilson</td>
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<td>Other</td>
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<td>Abbreviations: CIT, time between start cold perfusion in the donor and end of cold preservation of the liver graft; WIT, time between the end of cold ischemic preservation of the liver and start of reperfusion in the recipient.</td>
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Statistical Analysis. Statistics were performed using SPSS Version 12.0 for Windows (SPSS Inc., Chicago, IL). All data are expressed as mean values and standard error of the mean (SEM). Differences were analyzed using the Mann Whitney U test, Pearson correlation test, and Wilcoxon signed rank test where appropriate. P values were two-tailed and statistically significant at a level of less than .05.

**Results**

**Restoration of Biliary Lipid Secretion After Successful Liver Transplantation.** Twenty-four patients who received a liver transplant participated in this study. Bile flow increased progressively during the first postoperative week, reflecting adequate recovery of hepatobiliary function of the liver after transplantation (Fig. 1A). Biliary bile salt secretion, known to be the main driving force for bile production, increased curvilinearly (Fig. 1B). Concomitantly, the biliary excretion rates of phospholipids and cholesterol significantly increased during the first postoperative week (Fig. 1C-D) and continued to rise during the second week.

When output rates of bile salts, phospholipids, and cholesterol were plotted against each other, strong positive correlations were found (Fig. 2A-C), as has been previously reported.29 When bile salt output approached a zero secretion rate, no phospholipid or cholesterol secretion was observed (Fig. 2A-B). Thus, it appears that hepatobiliary lipid secretion proceeds normally under posttransplantation conditions.

**Hepatic ABCG5 and ABCG8 Expression Does Not Correlate With Biliary Cholesterol Secretion in Patients After Liver Transplantation.** By using real-time PCR, levels of transporter mRNA were analyzed in control livers and in liver grafts before transplantation, 3 hours after resuming blood circulation of the transplant, and 1 week after the operation. Messenger RNA copy numbers of transporter genes were normalized to those of 18S rRNA.

Liver biopsies collected 1 week after transplantation were used to compare levels of ABCG5 and ABCG8 transporter expression with the biliary secretion of cholesterol. At this time, biliary lipid secretion was analyzed in detail.
Because of the assumption that ABCG5 and ABCG8 are half-transporters that have to dimerize to generate a functional protein, levels of mRNA of the two genes were directly compared. Although levels of ABCG8 mRNA expression were somewhat higher than those of ABCG5, strong positive correlations between the levels of ABCG5 and ABCG8 mRNA were found at all three time points, supporting a similar mode of regulation (Fig. 3A-C).

The levels of mRNA of two other major transporters, the bile salt transporter BSEP (gene symbol ABCB11) and the phospholipid translocator MDR3 (gene symbol ABCB4) were also determined, as summarized in Fig. 4. Except for minor, nonsignificant changes, levels of BSEP, MDR3, ABCG5, and ABCG8 mRNA in liver transplants before operation and after 3 hours of recirculation did not differ from expression levels in control livers. One week after transplantation, BSEP expression was significantly increased compared with control livers and liver grafts before transplantation and after reperfusion (Fig. 4). Levels of MDR3, ABCG5, and ABCG8 mRNA at 1 week were unchanged compared with control values. Levels of mRNA in liver grafts varied between individuals (minimal and maximal levels of mRNA at 1 week: 0.28-3.43, 0.26-2.85, and 0.26-4.31 for MDR3, ABCG5, and ABCG8, respectively). Within individual liver grafts, mRNA levels of MDR3, ABCG5, and ABCG8 did not
Because phospholipids may drive cholesterol secretion and MDR3 controls phospholipid output,\textsuperscript{30,31} relationships between levels of hepatic expression of ABCG5 and ABCG8 toward MDR3 were analyzed. At all 3 time points, strong positive correlations were found between levels of ABCG5/MDR3 and ABCG8/MDR3 expression (Fig. 5A-C).

To segregate effects of phospholipid secretion on cholesterol secretion, the ratios of ABCG5 and ABCG8 to MDR3 expression were calculated and were plotted as a function of the ratio of cholesterol to phospholipid excretion, as previously shown by Kosters et al.\textsuperscript{20} In contrast with the strong positive relationship between the normalized Abcg5 and Abcg8 values and the normalized biliary cholesterol output in (genetically modified) mice,\textsuperscript{20} we could not find such a relationship in patients who had received a liver transplant (Fig. 6). Also, when the biliary secretion of cholesterol was not corrected for the secretion of phospholipid, no relationship between ABCG5/G8 and absolute biliary cholesterol secretion was observed ($r = 0.21$, $P = .31$, and $r = 0.05$, $P = .80$, for ABCG5 and ABCG8, respectively). As expected, MDR3 expression correlated well with the biliary phospholipid concentration ($r = 0.42$, $P = .03$), but not with the concentration of cholesterol ($r = 0.25$, $P = .21$) in the bile.

In search for an explanation for the unexpected lack of correlation between cholesterol output and ABCG5/G8 expression, biliary bile salt composition was analyzed to allow calculation of the “hydrophobicity index.”\textsuperscript{26} Although, in general, relatively hydrophobic bile salts are
more effective in driving biliary cholesterol secretion, no relationship between the hydrophobicity index and absolute or normalized biliary cholesterol output was found in bile collected 1 week after OLT (Fig. 7).

In search for possible alternative pathways of hepato-biliary cholesterol secretion, levels of SR-BI and ABCA1 mRNA were analyzed.32,33 Sehayek et al.32 have suggested that SR-BI may directly promote the excretion of cholesterol into the bile. In transgenic mice overexpressing the human ABCA1 gene, the biliary secretion of cholesterol was found to be facilitated.33 Neither SR-BI nor ABCA1 expression, however, showed any relationship with absolute or normalized biliary cholesterol secretion at 1 week after undergoing liver transplantation (r = 0.19, P = .35 and r = −0.16, P = .44, for SR-BI and ABCA1, respectively). Surprisingly, a strong linear relationship between levels of hepatic SR-BI and ABCA1 expression was observed (r = 0.93, P < .0001).

**Discussion**

In the current study, we followed the recovery of bile formation after successful human OLT and assessed the relationship between levels of hepatic expression of the ATP-binding cassette (ABC) half-transporters ABCG5/ABCG8 and the secretion of cholesterol into bile. A vast body of evidence has been generated demonstrating that, in mice, Abcg5/Abcg8 are of crucial importance in the control of biliary secretion of sterols.5,6,34 The main finding of this study was that we were not able to detect a relationship between the normalized hepatic expression of ABCG5 and ABCG8 and the normalized biliary output of cholesterol in humans (Fig. 5), whereas such a relationship is present in rodents.

After transplantation, bile flow recovered gradually. Bile flow is mainly driven by active biliary bile salt secretion.29 The recovery of bile flow therefore is likely a consequence of normalization of the circulating bile salt pool. In parallel with the recovery of bile salt secretion, cholesterol secretion and phospholipid output increased and were found to be tightly related to each other, as expected.29 The observed recovery of bile flow and the relationships between biliary secretion rates of bile salts,
phospholipids, and cholesterol in our study were similar to those previously described by others.35,36

Changes in bile formation were not associated with marked alterations in hepatic transporter expression: only levels of mRNA of the bile salt transporter BSEP (gene symbol ABCB11) were slightly, but significantly, increased at 1 week after OLT compared with levels in control livers and liver grafts before transplantation and 3 hours after reperfusion. The levels of mRNA of the phospholipid translocator MDR3 (gene symbol ABCB4) and ABCG5/G8 remained stable between the different time points. We could clearly demonstrate a strong positive relationship between ABCG5 and ABCG8 expression levels at the 3 time points studied (Fig. 3A-C), suggesting a tight coupling between the two transporter genes in human liver. This relationship supports the concept that ABCG5 and ABCG8 are partners in the generation of a functional transporter.14 The parallel expression of ABCG5/G8 also supports the perception that both genes are coordinately regulated. The nuclear receptors liver receptor homolog-1 (LRH-1) and liver X receptor (LXR) have been proposed as major regulatory transcription factors for ABCG5/G8.37,38 LRH-1 and LXRα are known to regulate the expression of many key enzymes in lipid metabolism.18,38 Using electrophoretic mobility shift assays, Freeman et al.38 recently found an LRH-1–specific binding motif in the ABCG5/G8 intergenic region. By mutating this LRH-1 binding site, a more than 7-fold reduction in ABCG5/G8 promoter activity was observed.38 Repa et al.37 showed that ABCG5/G8 are direct target genes of the oxysterol-activated nuclear receptor LXRα.

Phospholipids are required for biliary cholesterol secretion. Mice lacking Mdr2 (Abcb4, the murine homologue of MDR3/ABC4) have low or undetectable cholesterol concentrations in the bile.6,30 Because of the close coupling between cholesterol and phospholipid secretion, the ratio of cholesterol to phospholipid secretion, the ratio of cholesterol to phospholipid secretion (normalized cholesterol output) provides a functionally relevant reflection between cholesterol and phospholipid secretion, the normalization biliary cholesterol output.26 When corrected for the biliary hydrophobicity index, however, no relationship could be found. We speculate that in humans the actual canalicular transport step may be mediated by ABCG5/G8, but that this step is not rate-controlling in the overall process. Alternatively, it may be that in humans alternative, ABCG5/G8-independent processes are involved. In mice fed a diosgenin-containing diet, causing a dramatic ~15-fold increase in biliary cholesterol secretion, Kosters et al.20 observed that levels of hepatic Abcg5/g8 expression remained unaffected. By determination of the flux control coefficient of murine Abcg8, they could demonstrate that the rate control by Abcg8 in mediating biliary cholesterol efflux was limited.41 Flux control coefficient values of Abcg8 in mice varied from 50% at low rates of cholesterol secretion and increased to up to approximately 73% at “Vmax”.41 Yu et al.21 subsequently confirmed that diosgenin feeding in mice increases biliary cholesterol concentrations without changing hepatic Abcg5/g8 mRNA and protein levels. These authors also confirmed that the presence of Absc5 and Abcg8 is required for the stimulatory effect of diosgenin,21 as was shown earlier by Kosters et al.,41 who demonstrated an absence of an increase in biliary cholesterol secretion in diosgenin-treated Absc5−/− mice. The nuclear receptor pregnane X receptor (PXR) may be the mediator of diosgenin-induced biliary hypersecretion of cholesterol. In rats, the PXR ligand pregnenolone-16α-carbonitrile has been shown to increase secretion of biliary cholesterol.42 Increases in biliary cholesterol concentrations on diosgenin feeding were markedly attenuated in Pxr−/− mice.21 Biliary cholesterol secretion therefore might be facilitated by certain PXR target genes21 that may be involved in modulation of the activity of Abcg5/g8 transporters or may constitute an alternative pathway of canalicular cholesterol excretion.

The scavenger receptor class B type I (SR-BI) has been suggested to modulate cholesterol secretion into bile.32 SR-BI–deficient mice43,44 and SR-BI transgenic mice45 showed reduced and markedly enhanced biliary cholesterol secretion, respectively. SR-BI is known to play a role in selective high-density lipoprotein (HDL) cholesterol uptake at the basolateral hepatocyte membrane, but Se-
hayek et al. hypothesized that SR-BI also may be directly involved in biliary cholesterol secretion. We, however, did not observe any correlation between levels of SR-BI mRNA and secretion of cholesterol into the bile, indicating no rate-controlling function for SR-BI in hepatobiliary cholesterol flux in humans. Finally, although hepatobiliary cholesterol secretion has been shown to be facilitated in transgenic mice overexpressing human ABCA1, we found no correlation between levels of hepatic ABCA1 mRNA and biliary secretion of cholesterol. We did find an extremely strong correlation between levels of hepatic SR-BI and ABCA1 mRNA, suggesting a coordinate regulation of both genes that are crucial in (hepatic) HDL metabolism. The nature of this regulation, indicative for a functional link between selective HDL cholesterol uptake and pre-BHDL formation, is under investigation.

Although in Abcg5−/−/Abcg8−/− mice the levels of biliary cholesterol are extremely low compared with normal wild-type mice, the secretion of cholesterol and other sterols in bile is reduced by only approximately 50% in patients with sitosterolemia compared with healthy individuals. The residual biliary sterol secretion in sitosterolemia patients, and the absence of any relationship between hepatic ABCG5/G8 expression and biliary cholesterol secretion in our study, supports the notion that additional transport mechanisms may operate in parallel to ABCG5 and ABCG8 in mediating canalicular efflux of cholesterol in humans.

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References