The ABC of cholesterol transport

Plösch, Torsten

CHAPTER 3

Sitosterolemia in ABC-transporter G5-deficient mice is aggravated on activation of the liver-X-receptor

Torsten Plösch[1]
Vincent W. Bloks[1]
Yuko Terasawa[2]
Sara Berdy[2]
Karen Siegler[2]
Fjodor van der Sluijs[1]
Ido P. Kema[1]
Albert K. Groen[3]
Bei Shan[2]
Folkert Kuipers[1]
Margrit Schwarz[2]

[1] Center for Liver, Digestive, and Metabolic Diseases, University Hospital Groningen, Groningen, The Netherlands
[2] Tularik Inc., South San Francisco, CA, USA
[3] Center for Experimental Hepatology, Academic Medical Center, Amsterdam, The Netherlands

Adapted from *Gastroenterology (2004) 126:290-300.*

Summary

Mutations in either ATP-binding cassette (ABC) half-transporter G5 or G8 cause sitosterolemia. It has been proposed that ABCG5/ABCG8 heterodimers mediate secretion of plant sterols and cholesterol by hepatocytes into bile and their efflux from enterocytes into the intestinal lumen. To test whether deficiency of ABCG5 alone is sufficient to induce sitosterolemia, Abcg5-null mice were generated and characterized with respect to sterol metabolism. Abcg5-deficiency was associated with strongly elevated plasma levels of β-sitosterol (37-fold) and campesterol (7.7-fold) as well as reduced plasma cholesterol concentrations (-40%). Retention of orally administered [3H]ß-sitosterol in the intestinal wall (+ 550%) and plasma (+640%) was higher in Abcg5-null mice than in wild-type controls. Surprisingly, high plasma β-sitosterol and campesterol concentrations were even further elevated in Abcg5-null mice upon treatment with the synthetic LXR agonist T0901317 (0.015% dietary supplementation, 10 days), whereas these concentrations were reduced by ~75% in wild-type mice. Both cholesterol and phospholipid concentrations in gallbladder bile were decreased but, unexpectedly, cholesterol/phospholipid ratios were unchanged in the absence of Abcg5 and increased in both genotypes upon LXR activation. Hepatic expression of Abcg8 was reduced by about 35% in Abcg5-deficient mice when compared to controls. No compensatory overexpression of other ABC transporters potentially involved in hepatic cholesterol trafficking was observed on mRNA level. Our data show that disruption of the Abcg5 gene alone is sufficient to cause hyperabsorption of dietary plant sterols and sitosterolemia in mice, whereas the ability to secrete cholesterol into bile is maintained.

Introduction

The search for proteins involved in transport of cholesterol and related sterols across membranes during the course of their hepatobiliary secretion and intestinal absorption has been the "holy grail" for many investigators during the past decades. The search appears to be over: a series of recent studies indicate that specific ABC transporters, *i.e.*, Abcg5 and Abcg8, are critically involved in both processes.[1-5] Mutations in the human genes encoding ABCG5 and ABCG8, half-transporters that are considered to heterodimerize into a functional transporter,[1] have been shown to cause the inherited disease sitosterolemia[1,3,6] which is associated with a reduced biliary secretion as well as with a strongly enhanced intestinal absorption of plant sterols (ß-sitosterol, campesterol) and cholesterol. The genes encoding these transporters are highly expressed in liver and intestine of both human and mice.[1,7] Disruption of both the *Abcg5* and *Abcg8* genes in mice resulted in a sitosterolemic phenotype and was associated with a ~90% reduction in cholesterol content of gallbladder bile.[5] Heterozygotes (*Abcg5*$^{+/-}$/*Abcg8*$^{+/-}$) showed approximately half of the biliary cholesterol concentration seen in control mice.[5] Overexpression of the human genes in transgenic mice[4] and pharmacological induction of endogenous *Abcg5* and *Abcg8* gene expression[8,9] both resulted in a very significant hypersecretion of biliary cholesterol. Intestinal ABCG5 and ABCG8 are supposedly involved in efflux of plant sterols from the enterocytes back into the intestinal lumen, thereby preventing their net intestinal absorption.[1,10] Based on the fact that sitosterolemia patients absorb cholesterol from the intestinal lumen at a very high rate, a role for ABCG5 and ABCG8 in the control of cholesterol absorption efficiency has been proposed.[1,3,6] Consistent with this hypothesis, cholesterol absorption was reduced in mice overexpressing both transporters[4] and in mice in which expression of the transporters was induced by pharmacological means.[8,9]

Combined, the data summarized above clearly support crucial roles for both ABCG5/Abcg5 and ABCG8/Abcg8 in control of human and murine sterol metabolism, in particular with respect to prevention of sterol accumulation in the body. However, a number of issues regarding both mode of action and physiological role of these transporters still need to be addressed. The first of these relates to the findings by Graf *et al.*,[11] who employed epitope-tagged ABCG5 and ABCG8 to demonstrate in an elegant series of *in vitro* studies that both proteins are indeed expressed at the apical surface of polarized hepatocytic cells. Exit of these proteins from the endoplasmic reticulum and their targeting to the plasma membrane required co-expression of both half-transporters. While these results suggest that heterodimer formation is essential for trafficking in hepatocytes, they raise the question whether this process is regulated in the same way in enterocytes. It is conceivable that the hepatoma cell line used in these experiments lacked the expression of other half-transporters which may potentially function as heterodimeric partners for either ABCG5 or ABCG8 in a tissue-specific manner. The second issue concerns the functional role of the putative transporter pair

in the process of cholesterol secretion into bile. In Abcg5/Abcg8-deficient mice, biliary cholesterol secretion was not completely abolished, and was induced by cholesterol feeding,[5] possibly indicating the existence of an Abcg5/Abcg8-independent pathway for biliary cholesterol secretion. Accordingly, in a recent study[12] we found that while hepatic *Abcg5/Abcg8* expression levels generally correlated with biliary cholesterol output rates in a variety of mouse models of cholesterol hypo- and hypersecretion, there are some models where this correlation does not hold true. Particularly, the well-known massive induction (14-fold) of cholesterol secretion in mice fed with the plant sterol diosgenin was not associated with any change in hepatic *Abcg5/Abcg8* mRNA or Abcg5 protein levels. A third open question concerns the physiological role of ABCG5 and ABCG8 in the intestine and in the maintenance of cholesterol homeostasis. In mice overexpressing Abcg5 and Abcg8, the rate of intestinal cholesterol absorption as measured with the sitostanol/cholesterol dual radioisotope method was only modestly decreased[4] and probably influenced by the high influx of biliary cholesterol in these animals.[13] In *Abcg5*$^{-/-}$/*Abcg8*$^{-/-}$ mice, on the other hand, the fractional absorption of plant sterols and cholestanol was found to be 2- to 3-fold increased while that of cholesterol was not significantly affected.[5]

Thus, to address these questions and to gain full insight into the mode(s) of action of Abcg5 and Abcg8 in liver and intestine, it is important to have systems available in which the expression of either transporter alone or in combination is varied independently in either liver or intestine. As a first step towards dissection of the complex interactions between Abcg5 and Abcg8 and their specific roles in cholesterol metabolism, Abcg5-deficient mice have been generated and characterized. The results of this study demonstrate that the absence of Abcg5 alone is sufficient to induce a sitosterolemic phenotype in mice which, surprisingly, was aggravated upon activation of the Liver X-receptor (LXR) by pharmacological means. Cholesterol absorption appeared to be unaffected in *Abcg5*-null mice but, in contrast to the situation in wild-type mice, was insensitive to the inhibitory actions of LXR activation. In addition, the cholesterol content of gallbladder bile from Abcg5-deficient mice was decreased to a much lesser extent as reported for *Abcg5/Abcg8* double knockouts and, when corrected for differences in phospholipid content, was indistinguishable from that in control mice. Interestingly, biliary cholesterol concentration increased to a similar extent upon LXR activation in *Abcg5*$^{-/-}$ and wild-type mice. These data indicate that formation of functional Abcg5/Abcg8 heterodimers may not be required for maintenance of all aspects of cholesterol homeostasis.

Materials and Methods

Animals and diets

Abcg5$^{-/-}$ mice were generated by Deltagen, Inc. (Redwood City, USA) using standard gene-targeting methods. A 87 bp fragment corresponding to a segment of exon 3[7] was replaced by a beta-galactosidase cDNA and a phosphoglycerate kinase promoter-driven neomycin resistance cassette in a targeting vector (Figure 1A). The construct was linearized and electroporated into embryonic stem cells derived from the 129/OlaHsd strain. Cells that harbored the desired mutation were identified by positive selection and injected into recipient C57BL/6J blastocysts to produce chimeras, which were used for the generation of F1 heterozygotes. F2 wild-type, heterozygous and homozygous mice were produced from F1 intercrosses in the expected Mendelian ratios. Mice were genotyped via PCR using allele-specific oligonucleotide primers (wild-type: CCAAATCCATGTGGTGTTTGGCCTC; GGCTGCTCA-GAAAAACGTCGCTCTG; knockout: GGCTGCTCAGAAAAACGTCGCTCTG; GACGAGT-

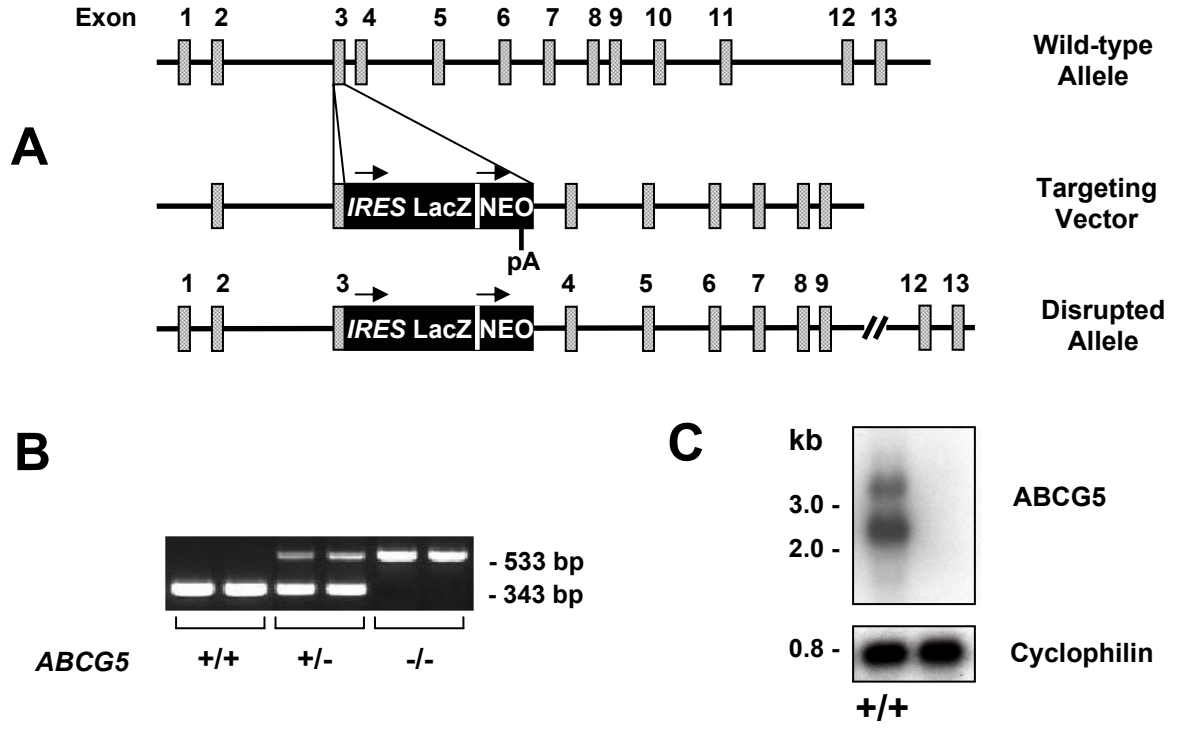

Figure 1: Targeted disruption of the *Abcg5* gene.

(A) Genomic organization of the wild-type allele, targeting construct, and disrupted allele. IRES, internal ribosomal entry site; LacZ, b-galactosidase cDNA; Neo, neomycin resistance cassette; pA, polyadenylation signal.

(B) Genotype analysis of genomic DNA from offspring of a heterozygote (*Abcg5*$^{+/-}$) mating. Genomic DNA was amplified via PCR using allele-specific primers. Products were separated by agarose gel electrophoresis.

(C) Northern blot analysis of hepatic RNA from wild-type and *Abcg5*$^{-/-}$ mice. PolyA^{+} RNA was isolated from liver and pooled within genotype groups (n=5). Aliquots (3 µg) were separated by gel electrophoresis, transferred to nylon membranes, and hybridized to a radiolabeled *Abcg5* probe representing exon 4 of the gene (1283 bp SacII/BamHI fragment (upper panel)). The filters were stripped and reprobed with a cDNA encoding rat *cyclophilin* (lower panel).

TCTTCTGAGGGGATCGATC). Male C57BL/6Jx129/OlaHsd mice with *Abcg5*$^{+/+}$ and *Abcg5*$^{-/-}$ genotypes were bred at Tularik Inc., South San Francisco, USA, and were housed in temperature-controlled rooms (23 °C) with 12 hours light cycling. Mice were fed *ad libitum* a cereal-based rodent diet (PicoLab Rodent Diet 5053, Purina Mills, Richmond, Indiana, USA) containing 4.7% fiber, 20% protein, 55% carbohydrates and 4.5% fat (soybean oil). All animals used for the experiments described below were male and 2-3 months of age. Experimental procedures were approved by the local Ethical Committee for Animal Experiments.

Experimental procedures

Groups of *Abcg5*$^{-/-}$ mice and wild-type littermates were fed either the powdered form of the diet described above or the same diet supplemented with the synthetic LXR-agonist T0901317 (0.015%, w/w) for 10 days. On day 7, mice were dosed by oral gavage with a mixture of [5,6-^{3}H]sitostanol (American Radiolabeled Chemicals, St. Louis, USA, 50 Ci/mmol) and [4-^{14}C]cholesterol (NEN, Boston, USA, 50 mCi/mmol) for measurement of intestinal cholesterol absorption efficiency by the dual-isotope fecal ratio method.[14,15] To determine the amount of radiolabeled dosing mixture administered to each animal, syringes were weighed before and after each oral gavage. Regurgitation did not occur. Feces from individually housed mice were collected during a 72 hr period from day 7 to day 10. On day 10, mice were fasted for 4 hours and euthanized with CO_2. The gallbladders were excised for bile collection and blood was collected in EDTA-containing tubes. Plasma aliquots were used for lipoprotein analyses and for determining the amount of [5,6-^{3}H]sitostanol absorbed. Livers were excised, rinsed with ice-cold phosphate-buffered saline (PBS) and weighed. The entire small intestine was excised, the contents flushed out with cold PBS and sectioned into three equal parts by length. Aliquots of liver and the intestinal segments were snap frozen in liquid nitrogen and stored at –80°C for mRNA isolation and biochemical analyses. Samples for evaluation by light microscopy were frozen in Tissue-Tek OCT compound (VWR International, Brisbane, USA) for cryosectioning, or fixed in neutral-buffered formaline for hematoxylin/eosin and Oil-red-O staining.

In a separate experiment, groups of wild-type and Abcg5$^{-/-}$ mice fed the same diet received an oral dose of [22,23-^{3}H]ß-sitosterol (American Radiolabeled Chemicals, 50 Ci/mmol, ~2 μCi/mouse) solubilized in corn oil (Sigma-Aldrich, St. Louis, USA). 24 h after dosing, mice were euthanized with CO_2 and blood, liver and mucosa of three intestinal segments were collected for quantification of radiolabeled sterols.

Analytical procedures

Biliary bile salt concentrations were measured enzymatically.[16] A commercially available kit was used for the determination of triglycerides (Roche, Mannheim, Germany). Plasma and hepatic cholesterol and plant sterol concentrations were determined by gas chromatography,

essentially as described by Kuksis *et al.*[17] Hepatic and biliary lipids were extracted according to Bligh and Dyer.[18] Phospholipids in bile and liver were determined as described by Böttcher *et al.*[19] Cholesterol in bile was measured according to Gamble *et al.*[20] Triglyceride content was analyzed as described above. Pooled plasma samples per group were used for lipoprotein separation by fast protein liquid chromatography (FPLC). Standard liver function tests (ALT, AST, LDH) were performed by routine clinical chemical procedures.

Feces were taken to a constant dry weight and homogenized to a powder. Aliquots of fecal powder were used for analysis of total neutral sterol and bile salt content according to Arca *et al.*[21] and Setchell *et al.*,[22] respectively. Separate aliquots were used for extraction of radiolabeled sterols and the calculation of fractional intestinal cholesterol absorption as described previously.[14]

RNA isolation and PCR procedures

Total RNA was extracted from frozen tissues with Trizol (Invitrogen, Carlsbad, USA) and quantified using Ribogreen (Molecular Probes, Eugene, USA). cDNA synthesis was performed according to Bloks *et al.*[23] Equal amounts of RNA from the three distinct parts of the small intestine were pooled prior to reverse transcription. Absence of wild-type *Abcg5* mRNA in liver tissue from *Abcg5*$^{-/-}$ mice was established by semiquantitative PCR using primers located in exon 2 and 4, respectively (TGTGCATCTTAGGCAGCTCA and CACAGTGAGGCTGCTCAGAA). Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector as previously described.[8] Primers were obtained from Invitrogen (Carlsbad, USA). Fluorogenic probes, labeled with 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA), were made by Eurogentec (Seraing, Belgium). Primers and probes used in these studies have been described elsewhere (*Srebp1a, Srebp1c, Srebp2, Lxra, Srb1, Acat1, Acat2, Hmgr, Cyp7a1, Cyp27, Abca1, Abcg5, Abcg8, Mdr2, Bsep*;[8] *beta actin*, *Ldlr*,[24] except *Lcat* (NM_008490; forward CTGGCTC-CTCAATGTGCTCTTC, reverse AGGCCGTGTGTGGTTACTGAGT, probe CCGCACAC-CACGCCCAAGGC) and *Cyp8b1* (NM_010012; forward AAGGCTGGCTTCCTGAGCTT, reverse AACAGCTCATCGGCCTCATC, probe CGGCTACACCAAGGACAAGCAGCA-AG). All expression data were subsequently standardized for *beta actin* which was analyzed in separate runs.

Statistics

All data are reported as mean ± S.D. Statistical analyses were performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, USA). Differences between genotypes within the same treatment group were evaluated using the Mann-Whitney-U-test. Comparison between untreated and treated wild-type and knockout mice were done by ANOVA with *Bonferoni*-correction. A p-value smaller than 0.05 was considered statistically significant.

Results

Generation of Abcg5-deficient mice

Targeted disruption of *Abcg5* was carried out by replacement of 87 bp of exon 3 with a beta-galactosidase cDNA and a neomycin resistance cassette (Figure 1A). Gene disruption was confirmed by PCR using genomic DNA from the offspring of heterozygote matings (page 67, Figure 1B). The deletion of 29 amino acids encoded by exon 3 was predicted to cause loss of Abcg5 protein expression and function by disrupting the conserved ATP-binding cassette signature motifs located in the N-terminal region of the protein.[7] Northern blotting analysis of hepatic RNA isolated from wild-type and *Abcg5*$^{-/-}$ mice revealed the presence of the expected two transcripts in livers of wild-type mice,[25] and the lack of any detectable transcript in the livers of *Abcg5*$^{-/-}$ mice (Figure 1C), indicating that the disruption strategy yielded a null allele of *Abcg5*. PCR analysis using primers located in exons 2 and 4 confirmed complete absence of wild-type *Abcg5* mRNA in liver tissue from knockout mice (data not shown). Noteworthy, hepatic and intestinal expression of *Abcg8* mRNA was also reduced and not responsive to T0901317 treatment (see below).

Abcg5$^{-/-}$ mice were healthy, fertile and developed normally under standard laboratory conditions. No gender bias was observed in the offspring. Routine histology of the liver and intestine as well as liver function tests were normal in the knockout animals, yet, liver size was slightly increased in *Abcg5*$^{-/-}$ mice in comparison to wild-type mice, *i.e.*, 5.78 ± 0.19 *vs.* 4.53 ± 0.18 g/100 g body weight ($p<0.05$).

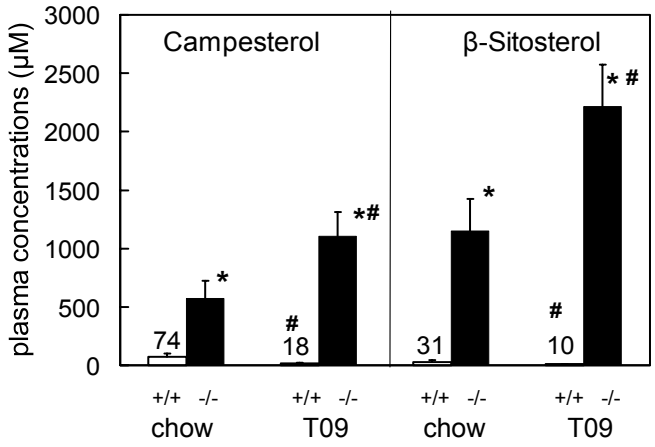

Figure 2: Plasma campesterol and ß-sitosterol levels in wild-type and *Abcg5*$^{-/-}$ mice.

Wild-type mice (open bars) and *Abcg5*$^{-/-}$ mice (filled bars) were fed a cereal-based rodent diet or the same diet supplemented with 0.015% (w/w) T0901317 for 10 days. Mice were euthanatized with CO_2 and blood was collected via the inferior *vena cava* in EDTA-containing tubes. Plant sterols were determined by gas chromatography as described in MATERIALS AND METHODS. n = 6-7 per group. * indicates significant difference from wild-type group, # from untreated group (ANOVA with Bonferoni-correction, $p<0.05$).

Sitosterolemia in Abcg5-deficient mice

Elevated plant sterol concentrations in plasma represent the hallmark of sitosterolemia in humans.[26,27] Accordingly, plasma levels of campesterol and β-sitosterol in Abcg5-deficient mice were elevated 7.7-fold and 37-fold, respectively, compared to those in wild-type mice (Figure 2). Furthermore, the less abundant plant sterols stigmasterol (5,22-cholestadien-24ß-ethyl-3ß-ol) and brassicasterol (5,22-cholestadien-24ß-methyl-3ß-ol) were increased in *Abcg5*$^{-/-}$ mice compared to wild-type mice, from non-detectable levels to 21.0 ± 5.4 μM and from 1.5 ± 1.7 μM to 7.3 ± 2.4 μM for stigmasterol and brassicasterol, respectively. Treatment of wild-type mice with the LXR agonist T0901317, known to induce hepatic and intestinal expression of *Abcg5* and *Abcg8* genes[8,25] (see also below), resulted in a significant lowering of plasma campesterol from 74.1 ± 28.4 to 17.7 ± 5.3 μM and of plasma β-sitosterol from 30.6 ± 12.3 to 9.7 ± 3.4 μM. In contrast, LXR activation in *Abcg5*$^{-/-}$ mice resulted in a further elevation of plasma campesterol (from 572 ± 153 to 1105 ± 207 μM, $p<0.05$) and ß-sitosterol (from 1148 ± 278 to 2215 ± 360 μM, $p<0.05$) levels (Figure 2), as also reported very recently for *Abcg5/Abcg8* double knockout mice.[9] The ATP binding cassette transporter A1 (Abca1) is known to be strongly induced upon LXR activation.[8] To test whether Abca1, in addition to its role in maintenance of cholesterol homeostasis, is also involved in regulation of plasma plant sterol levels, we used a mouse model lacking a functional *Abca1* gene.[8] Figure 3 shows that plasma campesterol and β-sitosterol levels were strongly reduced in *Abca1*$^{-/-}$ mice in comparison to controls (panel A), even when normalized to total plasma lipid concentrations in these animals (panel B).

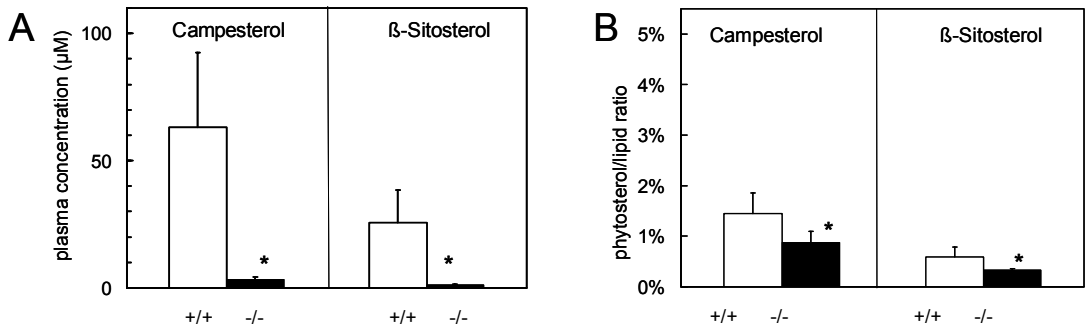

Figure 3: Plasma campesterol and ß-sitosterol levels in wild-type and *Abca1*$^{-/-}$ mice.

Wild-type mice (open bars) and *Abca1*$^{-/-}$ mice (filled bars) were maintained on standard laboratory chow as previously described by us.[8] Mice were anaesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and Diazepam (10 mg/kg), and blood was collected by cardiac puncture in EDTA-containing tubes. Plant sterols were determined by gas chromatography as described in MATERIALS AND METHODS. n=3 for *Abca1*$^{-/-}$ and n=10 for wild-type mice. * indicates significant difference from wild-type group (Mann-Whitney-U-test, $p<0.05$).

A, plasma concentration of campesterol and ß-sitosterol.

B, percentage of campesterol and ß-sitosterol, respectively, compared to total lipid (triglyceride + cholesterol).

Plant sterols accumulated not only in plasma, but also in the livers of *Abcg5*$^{-/-}$ mice. Relative sterol composition was 97.0%, 2.1% and 0.9% for cholesterol, campesterol, and ß-sitosterol, respectively, in wild-type mice, and 58.3%, 15.6%, and 26.1%, respectively, in *Abcg5*$^{-/-}$ mice. Hepatic triglyceride contents were similar in wild-type and *Abcg5*$^{-/-}$ mice, *i.e.*, 8.1 ± 4.0 and 11.4 ± 4.3 nmol/mg liver, respectively, indicating that the hepatomegaly observed in the latter group was not due to steatosis. Activation of LXR by T0901317 caused a similar degree of hepatic triglyceride accumulation in mice of both genotypes, *i.e.*, to 49.5 ± 3.9 and 47.5 ± 3.5 nmol/mg liver, respectively, probably due to induction of lipogenesis.[24,28]

To determine whether the lack of Abcg5 directly affects the uptake of plant sterols from the intestinal lumen, mice of both genotypes fed control diet were administered a bolus of [22,23-^{3}H]ß-sitosterol by oral gavage. Retention of radiolabeled ß-sitosterol was strongly increased in *Abcg5*$^{-/-}$ mice as compared to wild-type littermates. As summarized in Figure 4, the amount of radioactivity present in the mucosa of consecutive small intestinal sections (panel A) as well as in plasma (panel B) and liver (panel C) 24 hours after administration was significantly higher in *Abcg5*$^{-/-}$ than in wild-type mice.

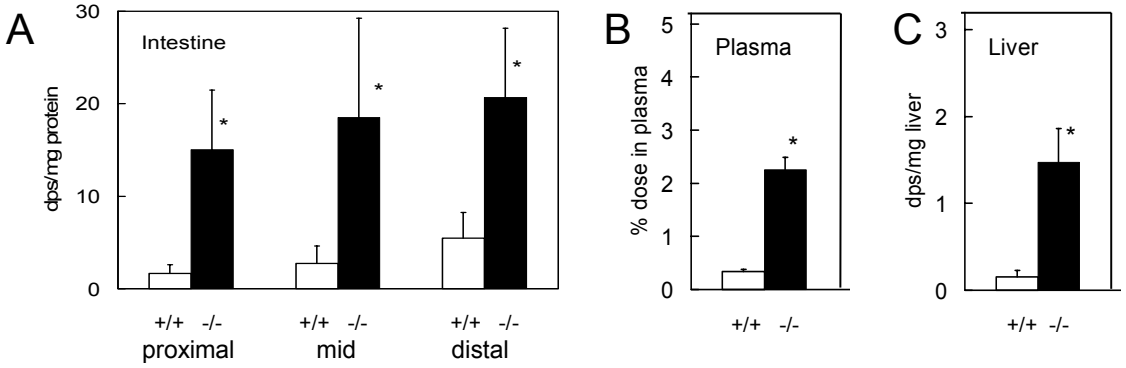

Figure 4: Retention of orally administered [^{3}H]sitosterol in intestinal mucosa, plasma and liver of wild-type and *Abcg5*$^{-/-}$ mice.

Wild-type mice (open bars) and *Abcg5*$^{-/-}$ mice (filled bars) fed a cereal-based rodent diet were orally dosed with [22,23-^{3}H]β-sitosterol solubilized in corn oil as described in MATERIALS AND METHODS. After 24 h, tissues and blood were harvested and analyzed as described there. n = 6-7 per group. * indicates significant difference from wild-type group (Mann-Whitney-U-test, $p<0.05$).
A, retention in the intestinal mucosa.
B, plasma accumulation.
C, hepatic enrichment.

Cholesterol and bile acid metabolism in Abcg5-deficient mice

Sitosterolemia is associated with normal or slightly elevated plasma cholesterol levels in humans.[26,27] In *Abcg5*$^{-/-}$ mice fed a cereal-based rodent diet, plasma total cholesterol levels as measured by gas chromatography were slightly reduced in comparison to those in wild-type littermates, while plasma triglycerides tended to be elevated (Table I). Excess triglycerides in

Table I: Plasma cholesterol and triglyceride concentrations in wild-type and *Abcg5*$^{-/-}$ mice.

		Cholesterol (mM)	Triglycerides (mM)
Control diet	wild-type	3.4 ± 0.8	1.3 ± 0.3
	Abcg5$^{-/-}$	2.0 ± 0.5 *	2.2 ± 0.7 *
T0901317	wild-type	8.0 ± 1.6 #	0.9 ± 0.4
	Abcg5$^{-/-}$	4.2 ± 0.7 *#	1.3 ± 0.3 #

Wild-type mice and *Abcg5*$^{-/-}$ mice were fed a cereal-based rodent diet or the same diet supplemented with 0.015% (w/w) T0901317 for 10 days. Mice were terminated with CO_2 and blood was collected via the inferior *vena cava* in EDTA-containing tubes. Triglycerides were measured enzymatically, cholesterol was determined by gas chromatography as described in MATERIALS AND METHODS. n=6-7 per group. * indicates significant difference from wild-type group, # from untreated group (ANOVA with Bonferoni-correction, $p<0.05$).

Abcg5$^{-/-}$ plasma were carried predominantly in VLDL-sized lipoprotein fractions, as revealed by FPLC analysis (*data not shown*). LXR activation resulted in a significant increase in plasma total cholesterol levels in mice of both genotypes, while triglyceride levels showed a decrease in *Abcg5*$^{-/-}$ mice (Table I).

Expression of genes involved in hepatocellular cholesterol homeostasis was quantified by realtime PCR (page 74, Table II). Steady state mRNA levels of genes encoding Srebp1a, 1c, 2 and LXRα, (oxy) sterol-regulated transcription factors that control cholesterol and lipid metabolism, did not differ between wild-type and *Abcg5*$^{-/-}$ mice: *Srebp1c* expression was induced upon LXR activation in both groups. Levels of *Hmgr* and *Ldlr* mRNA were lower in the liver of *Abcg5*$^{-/-}$ mice than in those from wild-type mice when animals were kept on a standard rodent diet, indicative for reduced cholesterol synthesis and LDL receptor-mediated lipoprotein uptake. Expression of genes that control key steps in bile salt biosynthesis (*i.e.*, *Cyp7a1*, *Cyp27*, *Cyp8b1*), a major route for cholesterol removal, did not differ between the groups, irrespective of genotype or dietary treatment.

To test whether lack of a functional *Abcg5* gene is associated with altered cholesterol absorption efficiency, the rate of intestinal cholesterol absorption was measured in wild-type and knockout mice using the fecal dual-isotope ratio method. Irrespective of genotype, sitostanol was only marginally absorbed as judged by [^{3}H] present in plasma 3 days after dosing. Assuming a total plasma volume of 4.7 ml per 100 g body weight, we determined the uptake of sitostanol to be 0.1% in wild-type and 0.3% in *Abcg5*$^{-/-}$ mice. We therefore rationalized that it is valid to use this sterol as a non-absorbable marker under these conditions. Figure 5 shows that the calculated fractional cholesterol absorption rate was 49.9 ± 5.5 % in wild-type mice and 50.4 ± 7.9 % in Abcg5-deficient mice. LXR activation resulted

Table II: Hepatic mRNA expression levels in wild-type and *Abcg5*$^{-/-}$ mice measured by real-time RT-PCR.

	Wild-type	*Abcg5*$^{-/-}$	Wild-type	*Abcg5*$^{-/-}$
mRNA	control diet		T0901317	
Srebp1a	1.00 ± 0.21	0.79 ± 0.11	1.18 ± 0.27	0.76 ± 0.14 *
Srebp1c	1.00 ± 0.14	0.89 ± 0.26	2.89 ± 1.05	1.68 ± 0.58 *
Srebp2	1.00 ± 0.08	0.82 ± 0.20	0.90 ± 0.16	0.72 ± 0.09 *
Lxra	1.00 ± 0.18	0.98 ± 0.22	0.95 ± 0.26	0.89 ± 0.09
Ldlr	1.00 ± 0.16	0.80 ± 0.20 *	1.23 ± 0.28	0.83 ± 0.11 *
Srb1	1.00 ± 0.27	0.83 ± 0.33	0.87 ± 0.11	0.81 ± 0.13
Hmgcr	1.00 ± 0.18	0.59 ± 0.31 *	1.20 ± 0.46	0.55 ± 0.13 *
Cyp7a1	1.00 ± 0.57	1.70 ± 0.87	1.07 ± 0.23	2.71 ± 0.91 *
Cyp27	1.00 ± 0.28	1.11 ± 0.39	1.01 ± 0.24	1.14 ± 0.16
Cyp8b1	1.00 ± 0.11	1.31 ± 0.73	0.82 ± 0.26	1.25 ± 0.39 *
Abcg5	1.00 ± 0.35	0.01 ± 0.01 *	2.97 ± 0.46	0.09 ± 0.04 *
Abcg8	1.00 ± 0.33	0.67 ± 0.22 *	1.82 ± 0.40	1.04 ± 0.18 *
Abcg1	1.00 ± 0.14	1.00 ± 0.38	2.31 ± 0.48	1.83 ± 0.20
Abcg2	1.00 ± 0.21	1.22 ± 0.28	1.53 ± 0.38	1.32 ± 0.12
Abca1	1.00 ± 0.18	1.07 ± 0.23	1.61 ± 0.18	1.39 ± 0.17
Abcb4	1.00 ± 0.12	0.97 ± 0.26	1.13 ± 0.12	1.19 ± 0.18
Abcb11	1.00 ± 0.19	0.88 ± 0.16	0.98 ± 0.16	0.88 ± 0.18

Wild-type mice and *Abcg5*$^{-/-}$ mice were fed a cereal-based rodent diet or the same diet supplemented with 0.015% (w/w) T0901317 for 10 days. After termination with CO_2, livers were removed and processed as described in MATERIALS AND METHODS. mRNA expression was measured by TaqMan real-time PCR as described there. All data were normalized to *beta-actin*. For *Abcg5*, less than 10% remaining, putatively inactive, mRNA was detectable in *Abcg5*$^{-/-}$ mice as the PCR-primers are located outside the affected exon. n = 6-7 per group. * indicates significant difference from wild-type group (Mann-Whitney-U-test, $p<0.05$).

in a marked reduction of cholesterol absorption to 22.2 ± 8.6 % in wild-type mice, but had no effect in Abcg5-deficient mice (48.9 ± 9.6 %). Consistent with these data, fecal neutral sterol excretion was similar in wild-type and *Abcg5*$^{-/-}$ mice (98.1 +/- 137.3 *vs.* 75.8 +/- 93.9 µmol/3d/100 g body weight). Treatment with T0901317 tended to increase fecal neutral sterol excretion in wild-type, but not in *Abcg5*$^{-/-}$ mice (150.7 +/- 99.2 *vs.* 82.4 +/- 73.9 µmol/3d/100 g body weight). Analysis of expression of genes potentially involved in intestinal cholesterol absorption revealed that mRNA levels of *Abca1* were higher in the intestine of *Abcg5*$^{-/-}$ mice as compared to wild-type mice and were induced to a similar extent upon LXR activation in mice of both genotypes (Table III). In contrast, intestinal *Abcg8* mRNA levels were lower in

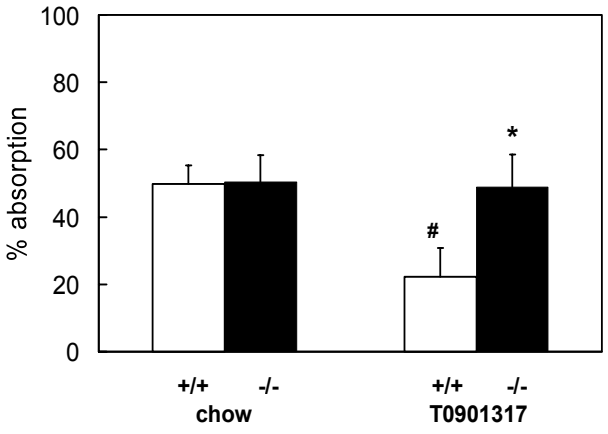

Figure 5: Fractional cholesterol absorption in wild-type and *Abcg5*$^{-/-}$ mice.

Wild-type mice (open bars) and *Abcg5*$^{-/-}$ mice (filled bars) were fed a cereal-based rodent diet or the same diet supplemented with 0.015% (w/w) T0901317 for 10 days. At day 7, mice were orally dosed with a [^{3}H]sitostanol/[^{14}C]cholesterol mixture in corn oil for measurement of intestinal cholesterol absorption as described in MATERIALS AND METHODS. n = 6-7 per group. * indicates significant difference from wild-type group, # from untreated group (ANOVA with Bonferoni-correction, $p<0.05$).

Abcg5$^{-/-}$ mice and were not induced upon T0901317-treatment. Expression of *Acat2*, involved in cholesterol esterification in the enterocyte and thereby essential for effective cholesterol absorption, did not differ between the groups. Likewise, similar expression levels of *Hmgr* mRNA indicated that endogenous intestinal cholesterol synthesis was likely not affected by the absence of Abcg5 or by LXR activation.

Overexpression or deficiency of both Abcg5 and Abcg8 in mice was shown to be associated with, respectively, strongly increased or decreased cholesterol contents of gall-bladder bile. To evaluate whether the lack of a functional Abcg5 gene alone would have similar consequences, gallbladder bile was collected from standard diet-fed and from T0901317-treated wild-type and *Abcg5*$^{-/-}$ mice. The biliary concentration of bile salts (Figure

Table III: Intestinal mRNA expression levels in wild-type and *Abcg5*$^{-/-}$ mice measured by real-time RT-PCR.

	Wild-type	*Abcg5*$^{-/-}$	Wild-type	*Abcg5*$^{-/-}$
mRNA	control diet		T0901317	
Abca1	1.00 ± 0.28	1.61 ± 0.57 *	5.86 ± 1.05	4.81 ± 1.80
Abcg5	1.00 ± 0.35	0.10 ± 0.03 *	2.43 ± 0.26	0.16 ± 0.08 *
Abcg8	1.00 ± 0.29	0.49 ± 0.09 *	2.24 ± 0.36	0.51 ± 0.21 *
Hmgcr	1.00 ± 0.11	1.01 ± 0.18	0.91 ± 0.20	1.12 ± 0.16
Acat2	1.00 ± 0.15	1.26 ± 0.16 *	1.03 ± 0.13	1.26 ± 0.09 *
Srb1	1.00 ± 0.31	1.02 ± 0.11	1.40 ± 0.30	1.46 ± 0.27

Wild-type mice and *Abcg5*$^{-/-}$ mice were fed a cereal-based rodent diet or the same diet supplemented with 0.015% T0901317 for 10 days. After termination, the small intestine was removed, frozen and processed as described in MATERIALS AND METHODS. mRNA expression was measured by TaqMan real-time PCR as described there. All data were normalized to *beta-actin*. For *Abcg5*, about 10% remaining, putatively inactive, mRNA was detectable in *Abcg5*$^{-/-}$ mice as the PCR-primers used were located outside the affected exon. n = 6-7 per group. * indicates significant difference from wild-type group (Mann-Whitney-U-test, $p<0.05$).

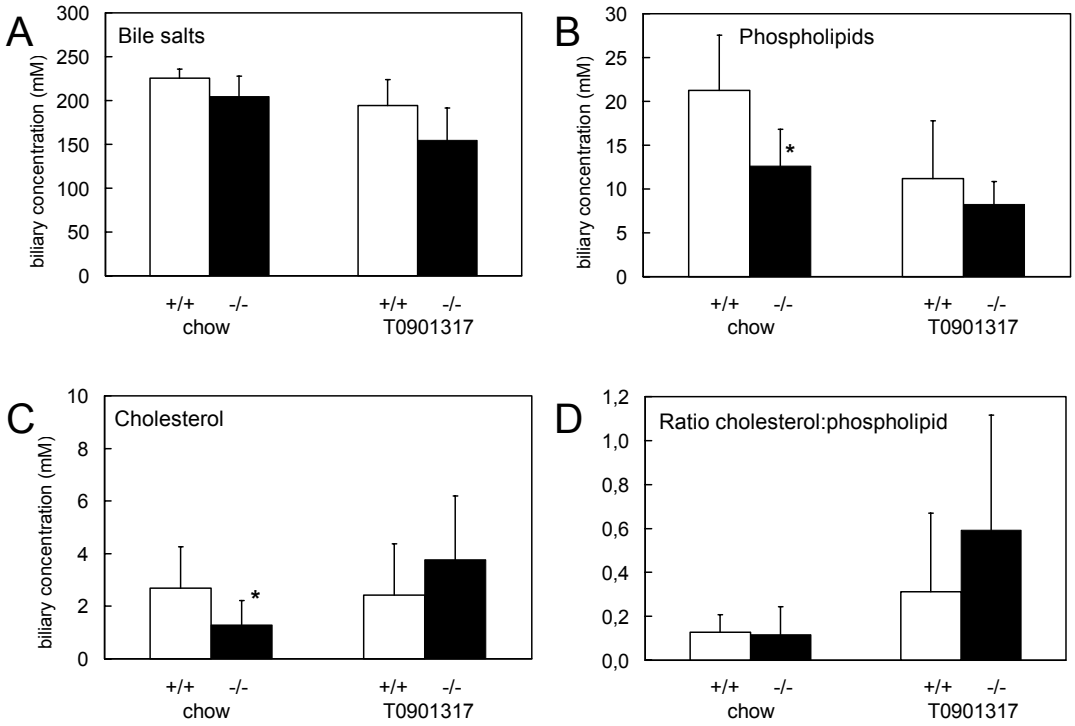

Figure 6: Biliary lipid composition of gallbladder bile of wild-type and *Abcg5*$^{-/-}$ mice.

Wild-type mice (open bars) and *Abcg5*$^{-/-}$ mice (filled bars) were fed a cereal-based rodent diet or the same diet supplemented with 0.015% (w/w) T0901317 for 10 days. On day 10, mice were fasted for 4 hours, followed by termination with CO_2. Bile was collected from the gallbladder. Analyses were performed as described in MATERIALS AND METHODS. n = 4-12 per group. * indicates significant difference from wild-type group (Mann-Whitney-U-test, $p<0.05$).

A, bile salt concentration. B, phospholipid concentration.
C, cholesterol concentration. D, ratio cholesterol/phospholipid.

6A) was unaffected by genotype, while concentrations of phospholipids (Figure 6B) and cholesterol (Figure 6C) were reduced to similar extents in *Abcg5*$^{-/-}$ mice. Hepatobiliary secretion of cholesterol is coupled to that of phospholipids in a process controlled by biliary bile salts.[29] In Figure 6D we therefore expressed cholesterol concentrations relative to those of phospholipids. Surprisingly, the relative concentration of cholesterol in gallbladder bile was not reduced in *Abcg5*$^{-/-}$ mice and tended to be induced to a similar extent upon LXR activation as in wild-type mice. Biliary bile salt composition, as analyzed by gas chromatography, was similar in *Abcg5*$^{-/-}$ and wild-type mice, with cholate (~55%) and ß-muricholate (~25%) being the major species.

Table II demonstrates that mRNA levels of *Abcg8* were reduced by 34% ($p<0.05$) in livers of *Abcg5*$^{-/-}$ mice compared to wild-type values, while expression of *Mdr2* (*Abcb4*) and *Bsep* (*Abcb11*), involved in phospholipid and bile salt transport, respectively, were not affected. Hepatic expression of other members of the Abcg subfamily of transporters, *i.e.*, *Abcg1* and *Abcg2*, nor those of *Abca1* (Table II), *Abca6*, *Abca7*, *Abca8* or *Abca9* (*data not shown*) were affected in livers of Abcg5-deficient mice. Treatment with the LXR agonist

T0901317 resulted in increased expression of *Abcg5* (+198%), *Abcg8* (+81%) and *Abca1* (+61%) (all $p<0.05$) in wild-type mice. This induction was less pronounced in livers of *Abcg5*$^{-/-}$ mice for *Abcg8* (+57%, $p<0.05$) and *Abca1* (+30%, NS).

Discussion

The results presented in this paper demonstrate that disruption of the *Abcg5* gene alone is sufficient to induce characteristic hallmarks of sitosterolemia in mice, *i.e.*, dramatically elevated concentrations of the plant sterols β-sitosterol and campesterol in plasma and in the liver.[26,27] *Abcg5*$^{-/-}$ mice appeared healthy and thrived normally when maintained under standard dietary conditions. At first glance, their phenotypic features were reminiscent of those recently reported for *Abcg5/Abcg8* double knockout mice.[5] Plasma and hepatic plant sterol levels were increased to a similar extent and plasma cholesterol levels were similarly reduced. Orally administered radioactive β-sitosterol accumulated to a much higher extent in intestinal mucosa, plasma and liver of *Abcg5*$^{-/-}$ mice than in wild-type controls, in full agreement with the proposed role of a functional Abcg5/Abcg8 heterodimer in prevention of plant sterol accumulation in the body.[1,10] Hepatic cholesterol levels were markedly lower in *Abcg5*$^{-/-}$ than in wild-type controls as were mRNA levels of the gene encoding HMGCoA reductase (*Hmgr*), consistent with reports by Yu *et al.*[5] in *Abcg5*$^{-/-}$/*g8*$^{-/-}$ mice. This suggests lower hepatic cholesterol synthesis rates in both models, although $[^3H]_2O$ incorporation into digitonin-precipitable hepatic sterols was unchanged in *Abcg5*$^{-/-}$/*g8*$^{-/-}$ mice.[5] Livers of *Abcg5*$^{-/-}$ mice were heavier than those from age- and sex-matched controls for reasons unknown so far.

To our surprise, plasma plant sterol concentrations were further elevated in *Abcg5*$^{-/-}$ mice upon treatment with the synthetic LXR agonist T0901317, a phenomenon also independently noted in *Abcg5*$^{-/-}$/*g8*$^{-/-}$ mice,[9] while this treatment resulted in a significant reduction of already low plasma plant sterol levels in wild-type mice. The latter is most likely a result of induction of intestinal and/or hepatic *Abcg5* and *Abcg8* expression,[8,25] leading to enhanced plant sterol efflux back into the intestinal lumen and accelerated sterol disposal from the liver. Considering the fact that plant sterol levels were strongly reduced in (untreated) *Abca1*$^{-/-}$ mice and that intestinal *Abca1* expression was significantly induced upon LXR activation in both wild-type and *Abcg5*$^{-/-}$ mice, it is tempting to speculate that Abca1, now known to be localized at the basolateral membrane of enterocytes,[30,31] plays a role in plant sterol absorption. In situations in which efflux back into the intestinal lumen is compromised, as is the case in *Abcg5*$^{-/-}$ mice, Abca1 may be able to accommodate an increased basolateral efflux of plant sterols, for instance to HDL which is known to carry the majority of plasma plant sterols.[32] This mechanism could partly explain the increased levels of plasma plant sterols in *Abcg5*$^{-/-}$ mice after LXR activation. Obviously, a separate series of experiments would be

required to test this hypothesis, which, if confirmed, would add another layer of complexity to the mechanisms that control selectivity of intestinal sterol absorption.

Noteworthy, hepatic and intestinal expression of *Abcg8* mRNA were reduced and not responsive to T0901317 treatment, suggesting the presence of *Abcg8*-promotor elements in the *Abcg5* gene. A putative LXR responsive element has been described[33] in intron 2 of the human *ABCG5* gene: LXR binding might be hampered by the insertion of the *lacZ-neo* cassette in the adjacent exon 3.

As an additional important finding we report that absence of *Abcg5* in mice does not affect the apparent efficiency of cholesterol absorption from the small intestine. Similar results have been reported for the *Abcg5*$^{-/-}$/*g8*$^{-/-}$ mice.[5] In both cases, the rate of cholesterol absorption was measured using the fecal dual-isotope ratio procedure. To validate this method in our experimental setting and for the particular genetic background of our model, we verified that the uptake of the "non-absorbable" marker [^{3}H]sitostanol is equally ineffective in wild-type and *Abcg5*$^{-/-}$ mice. This is consistent with the situation in sitosterolemia patients where similar values for fractional sterol absorption were found when either chromium oxide or sitostanol was used as fecal marker.[34] Our measurement is further supported by similar fecal neutral sterol excretion rates in knockout mice and controls. By contrast, a reduced neutral sterol output has been reported for *Abcg5*$^{-/-}$/*g8*$^{-/-}$ mice.[5] Although the actual biliary cholesterol secretion rate has not been determined for either of the two models, this difference may well be the consequence of biliary cholesterol entering the intestine at different rates. Increased disposal of biliary cholesterol has been suggested as an important determinant of cholesterol absorption efficiency in mice.[13] The fact that the absence of Abcg5 (or the Abcg5/Abcg8 heterodimer) causes hyperabsorption of plant sterols but does not affect absorption of cholesterol is consistent with the recent hypothesis by Igel *et al.*[10] suggesting that the process of discrimination between plant sterols and cholesterol probably occurs on the level of efflux from the enterocyte rather than on the level of uptake.

A key difference between *Abcg5*$^{-/-}$ and *Abcg5*$^{-/-}$/*g8*$^{-/-}$ mice became apparent when the composition of gallbladder bile was analyzed: cholesterol content was reduced by only 50% in *Abcg5*$^{-/-}$ compared to wild-type mice, while *Abcg5*$^{-/-}$/*g8*$^{-/-}$ mice are reported to almost lack biliary cholesterol.[5] In addition, this content tended to increase upon treatment with the LXR agonist in *Abcg5*$^{-/-}$ and wild-type mice alike. A number of possibilities exist to explain the relatively small effects of Abcg5-deficiency on gallbladder bile content. While we did not detect a compensatory overexpression of *Abcg8* or any of the other ABC transporters that potentially might facilitate biliary cholesterol secretion, *e.g.*, *Abca1*, *Abcg1* or *Abcb4*, Abcg8 might still act as a homodimer or heterodimerize with other partners, *e.g.*, Abcg1 or Abcg2, to maintain cholesterol excretion. This seems to be in apparent contradiction with data from Graf *et al.*[11] showing that co-expression of (epitope-tagged) Abcg5 and Abcg8 is required for apical targeting of the ABC-transporters in hepatoma cells, but it is conceivable that these cells lack the expression of other transporters which may serve as heterodimer partners for

Abcg8. Alternatively, mechanisms independent of both Abcg5 (and Abcg8) may be responsible for cholesterol excretion under certain conditions. Indications for Abcg5/g8-independent hepatobiliary cholesterol excretion came from our recent observation[12] that diosgenin specifically induces biliary cholesterol excretion 14-fold in mice without any effect on *Abcg5/Abcg8* expression.

The ratio cholesterol/phospholipid, reflecting hepatobiliary cholesterol secretion more accurately because of the close coupling between cholesterol and phospholipid secretion,[29] was virtually identical in wild-type and *Abcg5*$^{-/-}$ mice. This was highly surprising in view of the proposed crucial role of the Abcg5/Abcg8 heterodimer in the actual excretion process, which was based on the data on gallbladder bile obtained from *ABCG5/ABCG8* transgenics[4] and *Abcg5*$^{-/-}$/*g8*$^{-/-}$ mice.[5] At first glance, these data suggest that the 50% reduction of biliary cholesterol content is secondary to a reduced capacity to secrete phospholipids into bile, similar to the situation observed in Mdr2 (Abcb4)-deficient mice.[35,36] Yet, mRNA levels of *Mdr2* (*Abcb4*) were unchanged in *Abcg5*$^{-/-}$ mice. Interestingly, upon close inspection of data published on *Abcg5/g8*$^{-/-}$ mice, it appears that the phospholipid content of gallbladder bile tended to be decreased or was even significantly decreased in these animals.[4,5,9] It is therefore conceivable that, possibly due to the presence of excessive amounts of plant sterols in hepatic (plasma) membranes, defective Mdr2-mediated phospholipid secretion contributes to impaired cholesterol content of gallbladder bile in *Abcg5*$^{-/-}$ and *Abcg5*$^{-/-}$/*Abcg8*$^{-/-}$ mice.

Collectively, the data presented indicate that while Abcg5 is essential for adequate control of intestinal plant sterol absorption, it is not critical for the control of dietary cholesterol absorption under basal conditions. Furthermore, our data provide evidence that functional Abcg5/Abcg8 heterodimers are not absolutely required for hepatobiliary cholesterol secretion in mice. Clearly, more research is needed to define the molecular mechanism(s) of biliary cholesterol secretion and the role of Abcg5 and Abcg8 herein.

Acknowledgements

We thank Juul F.W. Baller, Renze Boverhof, Henk Wolters and Claude van der Ley for excellent technical assistance, and Yolanda Hatter, Laura Hoffman and Ali Motani for animal care and breeding. This study was supported by grant 902-23-193 from the Netherlands Organization for Scientific Research (NWO).

References

1. Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, Kwiterovich P, Shan B, Barnes R, Hobbs HH. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. *Science* 2000;290:1771-1775.
2. Lee MH, Lu K, Hazard S, Yu H, Shulenin S, Hidaka H, Kojima H, Allikmets R, Sakuma N, Pegoraro R, Srivastava AK, Salen G, Dean M, Patel SB. Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. *Nat Genet* 2001;27:79-83.
3. Lu K, Lee MH, Hazard S, Brooks-Wilson A, Hidaka H, Kojima H, Ose L, Stalenhoef AF, Mietinnen T, Björkhem I, Bruckert E, Pandya A, Brewer HB, Jr., Salen G, Dean M, Srivastava A, Patel SB. Two genes that map to the STSL locus cause sitosterolemia: genomic structure and spectrum of mutations involving sterolin-1 and sterolin- 2, encoded by ABCG5 and ABCG8, respectively. *Am J Hum Genet* 2001;69:278-290.
4. Yu L, Li-Hawkins J, Hammer RE, Berge KE, Horton JD, Cohen JC, Hobbs HH. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *J Clin Invest* 2002;110:671-680.
5. Yu L, Hammer RE, Li-Hawkins J, von Bergmann K, Lütjohann D, Cohen JC, Hobbs HH. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. *Proc Natl Acad Sci U S A* 2002;99:16237-16242.
6. Lee MH, Lu K, Hazard S, Yu H, Shulenin S, Hidaka H, Kojima H, Allikmets R, Sakuma N, Pegoraro R, Srivastava AK, Salen G, Dean M, Patel SB. Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. *Nat Genet* 2001;27:79-83.
7. Lu K, Lee MH, Yu H, Zhou Y, Sandell SA, Salen G, Patel SB. Molecular cloning, genomic organization, genetic variations, and characterization of murine sterolin genes Abcg5 and Abcg8. *J Lipid Res* 2002;43:565-578.
8. Plösch T, Kok T, Bloks VW, Smit MJ, Havinga R, Chimini G, Groen AK, Kuipers F. Increased Hepatobiliary and Fecal Cholesterol Excretion upon Activation of the Liver X Receptor Is Independent of ABCA1. *J Biol Chem* 2002;277:33870-33877.
9. Yu L, York J, von Bergmann K, Lütjohann D, Cohen JC, Hobbs HH. Stimulation of cholesterol excretion by LXR agonist requires ATP- binding cassette transporters G5 and G8. *J Biol Chem* 2003;278:15565-15570.
10. Igel M, Giesa U, Lütjohann D, von Bergmann K. Comparison of the intestinal uptake of cholesterol, plant sterols, and stanols in mice. *J Lipid Res* 2003;44:533-538.
11. Graf GA, Li WP, Gerard RD, Gelissen I, White A, Cohen JC, Hobbs HH. Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface. *J Clin Invest* 2002;110:659-669.
12. Kosters A, Frijters RJJM, Schaap FG, Vink E, Plösch T, Ottenhoff R, Jirsa M, de Cuyper IM, Kuipers F, Groen AK. Relation between hepatic expression of ATP-binding cassette transporters G5 and G8 and biliary cholesterol secretion in mice. *J Hepatol* 2003;38:710-716.
13. Sehayek E, Ono JG, Shefer S, Nguyen LB, Wang N, Batta AK, Salen G, Smith JD, Tall AR, Breslow JL. Biliary cholesterol excretion: a novel mechanism that regulates dietary cholesterol absorption. *Proc Natl Acad Sci U S A* 1998;95:10194-10199.

14. Schwarz M, Davis DL, Vick BR, Russell DW. Genetic analysis of intestinal cholesterol absorption in inbred mice. *J Lipid Res* 2001;42:1801-1811.
15. Wang DQ, Carey MC. Measurement of intestinal cholesterol absorption by plasma and fecal dual-isotope ratio, mass balance, and lymph fistula methods in the mouse: an analysis of direct versus indirect methodologies. *J Lipid Res* 2003;44:1042-1059.
16. Mashige F, Imai K, Osuga T. A simple and sensitive assay of total serum bile acids. *Clin Chim Acta* 1976;70:79-86.
17. Kuksis A, Myher JJ, Marai L, Little JA, McArthur RG, Roncari DA. Usefulness of gas chromatographic profiles of plasma total lipids in diagnosis of phytosterolemia. *J Chromatogr* 1986;381:1-12.
18. Bligh E, Dyer W. A rapid method of total lipid extraction and purification. *Can J Biochem Biophys* 1959;37:911-917.
19. Böttcher C, van Gent C, Pries C. A rapid and sensitive sub-micro phosphorus determination. *Anal Chim Acta* 1961;24:203-204.
20. Gamble W, Vaughan M, Kruth HS, Avigan J. Procedure for determination of free and total cholesterol in micro- or nanogram amounts suitable for studies with cultured cells. *J Lipid Res* 1978;19:1068-1070.
21. Arca M, Montali A, Ciocca S, Angelico F, Cantafora A. An improved gas-liquid chromatographic method for the determination of fecal neutral sterols. *J Lipid Res* 1983;24:332-335.
22. Setchell KD, Lawson AM, Tanida N, Sjovall J. General methods for the analysis of metabolic profiles of bile acids and related compounds in feces. *J Lipid Res* 1983;24:1085-1100.
23. Bloks VW, Plösch T, van Goor H, Roelofsen H, Baller JFW, Havinga R, Verkade HJ, van Tol A, Jansen PLM, Kuipers F. Hyperlipidemia and atherosclerosis associated with liver disease in ferrochelatase-deficient mice. *J Lipid Res* 2001;42:41-50.
24. Grefhorst A, Elzinga BM, Voshol PJ, Plösch T, Kok T, Bloks VW, van der Sluijs FH, Havekes LM, Romijn JA, Verkade HJ, Kuipers F. Stimulation of Lipogenesis by Pharmacological Activation of the Liver X Receptor Leads to Production of Large, Triglyceride-rich Very Low Density Lipoprotein Particles. *J Biol Chem* 2002;277:34182-34190.
25. Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding Cassette Sterol Transporters ABCG5 and ABCG8 by the Liver X Receptors alpha and beta. *J Biol Chem 2002*;277:18793-18800.
26. Salen G, Kwiterovich PO, Jr., Shefer S, Tint GS, Horak I, Shore V, Dayal B, Horak E. Increased plasma cholestanol and 5 alpha-saturated plant sterol derivatives in subjects with sitosterolemia and xanthomatosis. *J Lipid Res* 1985;26:203-209.
27. Björkhem I, Boberg KM, Leitersdorf E. Inborn Errors in Bile Acid Biosynthesis and Storage of Sterols Other than Cholesterol. In: Scriver C, Beaudet A, Sly W, and Valle D, eds. The Metabolic and Molecular Bases of Inherited Disease. 8 ed. New York: McGraw-Hill, 2001:2961-2988.
28. Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L, Schwendner S, Wang S, Thoolen M, Mangelsdorf DJ, Lustig KD, Shan B. Role of LXRs in control of lipogenesis. *Genes Dev* 2000;14:2831-2838.
29. Verkade HJ, Vonk RJ, Kuipers F. New insights into the mechanism of bile acid-induced biliary lipid secretion. *Hepatology* 1995;21:1174-1189.

30. Ohama T, Hirano K, Zhang Z, Aoki R, Tsujii K, Nakagawa-Toyama Y, Tsukamoto K, Ikegami C, Matsuyama A, Ishigami M, Sakai N, Hiraoka H, Ueda K, Yamashita S, Matsuzawa Y. Dominant expression of ATP-binding cassette transporter-1 on basolateral surface of Caco-2 cells stimulated by LXR/RXR ligands. *Biochem Biophys Res Commun* 2002;296:625-630.
31. Mulligan JD, Flowers MT, Tebon A, Bitgood JJ, Wellington C, Hayden MR, Attie AD. ABCA1 is essential for efficient basolateral cholesterol efflux during the absorption of dietary cholesterol in chickens. *J Biol Chem* 2003;278:13356-66.
32. Tilvis RS, Miettinen TA. Serum plant sterols and their relation to cholesterol absorption. *Am J Clin Nutr* 1986;43:92-97.
33. Remaley AT, Bark S, Walts AD, Freeman L, Shulenin S, Annilo T, Elgin E, Rhodes HE, Joyce C, Dean M, Santamarina-Fojo S, Brewer HB, Jr. Comparative genome analysis of potential regulatory elements in the ABCG5-ABCG8 gene cluster. *Biochem Biophys Res Commun* 2002;295:276-282.
34. Lütjohann D, Björkhem I, Beil UF, von Bergmann K. Sterol absorption and sterol balance in phytosterolemia evaluated by deuterium-labeled sterols: effect of sitostanol treatment. *J Lipid Res* 1995;36:1763-1773.
35. Smit JJ, Schinkel AH, Oude Elferink RP, Groen AK, Wagenaar E, van Deemter L, Mol CA, Ottenhoff R, van der Lugt NM, van Roon MA. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* 1993;75:451-462.
36. Oude Elferink RP, Ottenhoff R, van Wijland M, Frijters CM, van Nieuwkerk C, Groen AK. Uncoupling of biliary phospholipid and cholesterol secretion in mice with reduced expression of mdr2 P-glycoprotein. *J Lipid Res* 1996;37:1065-1075.