HEPATIC ABCG5 AND ABCG8 OVEREXPRESSION INCREASES HEPATOBILIARY STEROL TRANSPORT BUT DOES NOT ALTER AORTIC ATHEROSCLEROSIS IN TRANSGENIC MICE

Justina E. Wu¹, Federica Basso¹, Robert D. Shamburek¹, Marcelo J. A. Amar¹, Boris Vaisman¹, Gergeley Szakacs², Charles Joyce¹, Terese Tansey¹, Lita Freeman¹, Beverly J. Paigen³, Fairwell Thomas¹, H. Bryan Brewer, Jr.¹, Silvia Santamarina-Fojo¹

¹ Molecular Disease Branch, NHLBI, NIH, Building 10, Room 7N115, Bethesda, MD 20892, USA  
² Department of Agricultural Chemical Technology, Technology University of Budapest, Gellert ter 4, 1111, Budapest, Hungary  
³ The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609, USA

Send correspondence to: Silvia Santamarina-Fojo, MDB, NHLBI, NIH, Building 10, Room 7N115, Bethesda, MD 20892. Telephone: 301-496-5095; Fax: 301-402-0190; E-mail: silvia@mdb.nhlbi.nih.gov

Running Title: Contribution of hepatic ABCG5/G8 overexpression to sterol transport and atherosclerosis.

Key Words: ABCG5, ABCG8, sterol transporters, cholesterol absorption, atherosclerosis
SUMMARY

The individual roles of hepatic vs intestinal ABCG5 and ABCG8 in sterol transport have not yet been investigated. To determine the specific contribution of liver ABCG5/G8 to sterol transport and atherosclerosis, we generated transgenic mice that overexpress human ABCG5 and ABCG8 in the liver but not intestine (liver G5/G8-Tg) in three different genetic backgrounds: C57Bl/6, apoE-KO and LDLr-KO. Hepatic overexpression of ABCG5/G8 enhanced hepatobiliary secretion of cholesterol and plant sterols by 1.5-2 fold, increased the amount of intestinal cholesterol available for absorption and fecal excretion by up to 27% and decreased the accumulation of plant sterols in plasma by approximately 25%. However, it did not alter fractional intestinal cholesterol absorption, fecal neutral sterol excretion, hepatic cholesterol concentrations or hepatic cholesterol synthesis. Consequently, overexpression of ABCG5/G8 in only the liver had no effect on the plasma lipid profile, including cholesterol, HDL-C and nonHDL-C, or on the development of proximal aortic atherosclerosis in C57Bl/6, apoE-KO, or LDLr-KO mice. Thus, liver ABCG5/G8 facilitate the secretion of liver sterols into bile and serve as an alternative mechanism, independent of intestinal ABCG5/G8, to protect against the accumulation of dietary plant sterols in plasma. However, in the absence of changes in fractional intestinal cholesterol absorption, increased secretion of sterols into bile induced by hepatic overexpression of ABCG5/G8 was not sufficient to alter hepatic cholesterol balance, enhance cholesterol removal from the body or to alter atherogenic risk in liver G5/G8-Tg mice. These findings demonstrate that overexpression of ABCG5/G8 in the liver profoundly alters hepatic but not intestinal sterol transport, identifying distinct roles for liver and intestinal ABCG5/G8 in modulating sterol metabolism.

Nonstandard abbreviations: ATP-binding cassette transporter G5, ABCG5; ATP-binding cassette transporter G8, ABCG8; high density lipoproteins, HDL; low density lipoprotein receptor, LDLr; apolipoprotein, apo; knockout, KO; LXR, liver X receptor; cholesterol 7 alpha hydroxylase, CYP7; 3-hydroxymethylglutaryl coenzyme A reductase, HMG-CoA.
INTRODUCTION

Sitosterolemia, also known as phytosterolemia (1-9) is a rare genetic disorder characterized by elevated plasma and tissue levels of plant, shellfish and animal sterols and the development of tendon and tuberous xanthomas, hemolytic episodes, arthritis and premature coronary artery disease. The ATP-binding cassette (ABC) half-transporters ABCG5 and ABCG8 (ABCG5/G8) have been recently identified as the genes defective in sitosterolemia (10-12). The increased plasma and tissue levels of animal and plant sterols found in sitosterolemic patients has been attributed to enhanced absorption and decreased biliary excretion (1;2;2-4;6;8;13-16). Unlike cholesterol, sitosterol accumulation does not inhibit HMGCoA reductase activity in human monocyte-derived macrophages (17). The inadequate downregulation of HMGCoA reductase activity by plant sterols may promote foam cell formation and explain, in part, the increased risk of atherosclerosis in sitosterolemia (17).

Several lines of evidence implicate ABCG5/G8 in intestinal and biliary sterol transport. First, ABCG5/G8 are expressed in the liver and small intestine and, to a lesser degree, in the colon (10;12;18-20) and gallbladder (21) and have been shown to traffic to the apical surface of polarized hepatocyte WifB cells as heterodimers (22). Second, both in vitro and in vivo studies have shown that ABCG5/G8 gene expression is modulated by transcription factors previously implicated in lipid and sterol metabolism. Agonists of LXR, a nuclear hormone receptor involved in sterol metabolism, enhance ABCG5/G8 gene expression in rat hepatoma cells (19) and feeding mice cholesterol-rich diets upregulates ABCG5/G8 gene expression in the liver and intestine of normal but not LXRα-deficient mice (10). Deficiency of ABCG5 and ABCG8 in mice leads to increased intestinal cholesterol absorption and decreased gall bladder bile cholesterol concentration (23), a phenotype similar to that of patients with sitosterolemia. Conversely, overexpression of human ABCG5/G8 raised the gallbladder cholesterol concentrations by more than 5-fold and reduced dietary sterol absorption by 50% in transgenic mice (24). Finally, recent studies in mice and rats have demonstrated an association between ABCG5 and ABCG8 gene expression and changes in biliary cholesterol secretion and intestinal cholesterol absorption (25-27).
Thus, ABCG5/G8 are transporters that play a major role in the excretion of cholesterol and other sterols from the body.

Here we report the generation of transgenic mice that selectively overexpress hABCG5/G8 in the liver of C57Bl/6, apoE-KO, and LDLr-KO mice. We describe the contribution of liver ABCG5/G8 to hepatobiliary sterol transport and evaluate the effects of hepatic overexpression of these transporters on the development of atherosclerotic lesions. Our studies directly demonstrate a role for liver ABCG5/G8 in facilitating sterol secretion into bile and in reducing the plasma concentrations of potentially pro-atherogenic plant sterols in plasma. However, hepatic overexpression of ABCG5/G8 did not reduce fractional intestinal cholesterol absorption nor facilitate the elimination of cholesterol from liver G5/G8-Tg over that of control mice. Hence, despite increased hepatobiliary sterol secretion, overexpression of ABCG5/G8 in the liver did not alter hepatic cholesterol concentrations or induce compensatory changes in liver enzymes and in hepatic receptors that might lead to antiatherogenic changes in the plasma lipoprotein profile and decrease aortic atherosclerosis in C57Bl/6, apoE-KO and LDLr-KO mice. These findings identify separate functions for hepatic and intestinal ABCG5/G8, and suggest that increased expression of intestinal as well as hepatic ABCG5/G8 may be required to significantly increase the elimination of body cholesterol and modulate atherogenic risk.

**EXPERIMENTAL PROCEDURES**

*Generation of ABCG5/G8 Transgenic Mice*—A fully characterized and sequenced 140-kb BAC clone (BAC 26313; Incyte Genomics, Palo Alto, CA) encoding the complete ABCG5 and ABCG8 proteins (28) was used to generate transgenic mice in the C57Bl/6 background as described (29). Integration of the hABCG5/G8 genes was determined by dot blot hybridization analysis of genomic mouse tail DNA using full length hABCG5/G8 cDNA probes and confirmed by PCR using human-specific ABCG5/G8 primers (sequences available upon request). Several different mouse lines expressing hABCG5/G8 were identified and expanded for further studies. To generate apoE-KO or LDLR-KO mice overexpressing hABCG5/G8, transgenic mice were crossed with apoE-
KO or LDLr-KO mice in the C57Bl/6 background. All experiments were performed according to a research protocol approved by the Animal Care and Use Committee of the NHLBI, NIH.

**Diets**—Control and transgenic mice (2-5 months of age) were maintained on the NIH-31 rodent diet (Harlan Teklad, Madison WI), designated as the regular chow diet (RCD; 0.025% cholesterol, 0.05% sitosterol, 0.015% campesterol and 0.007% stigmasterol), or a 4% cholesterol diet (designated 4%C; 3.9% cholesterol, 0.04% sitosterol, 0.016% campesterol, and 0.009% stigmasterol) for a period of 10-12 weeks prior to analyses. To induce the formation of aortic lesions, C57Bl/6 control and transgenic mice were placed on the high-fat, high-cholesterol/cholate diet as described (30;31) and LDLr-KO mice were placed on a Western diet (0.2% cholesterol, 21% fat, <0.003% sitosterol, campesterol and stigmasterol). All diets were analyzed by Woodson-Tenent Laboratories (Memphis, TN) to confirm their sterol content. Daily food intake was measured in a representative group of 4 mice for each study group.

**RNA Isolation and Northern Blot Analysis**—Total RNA was isolated (Ultraspec Kit, Biotecx, Houston, TX) from age and sex matched mice as per manufacturer’s instructions. RNA (20-30µg) was subjected to Northern analysis by hybridizing with $^{32}$P-labelled cDNA probes that detect either: i) both human and mouse ABCG5 and ABCG8 mRNA (human ABCG5, full length; human ABCG8, bp-1047 to 2561); ii) only mouse ABCG5 and ABCG8 mRNA (mouse ABCG5, bp-2097 to 2287; mouse ABCG8, bp-2122 to 2236); and iii) only human ABCG5 and ABCG8 mRNA (human ABCG5, bp-2099 to 2320; human ABCG8, bp-2112 to 2244). Other probes used include Cyp-7α hydroxylase (bp-719 to 1738), LDL receptor (LDLr), HMGCoA reductase, ABCA1, and β-actin as described (32;33). $^{32}$P-labelled mRNA signals were quantified and normalized to β-actin. For RT-PCR, total RNA (2 µg) was reverse-transcribed using the SuperScriptII First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with oligo(dT) primers according to manufacturer’s instructions. Human and mouse ABCG5/G8 mRNA levels were quantitated by real-time quantitative RT-PCR using the LightCycler FastStart DNA Master SYBR Green kit (Roche Biochemicals, Indianapolis, IN) and a LightCycler, with mouse succinate dehydrogenase for normalization (Mouse SDHA-
Certified LUX™ Primer Set, Invitrogen). Sequences of gene-specific primers are available upon request. Specific product formation was verified by melting-curve analysis and gel electrophoresis.

**Analyses of Plasma Lipids**—Plasma was obtained after a 4-hour fast and lipid levels were determined as previously described (29;32). HDL-cholesterol (HDL-C) was determined as the cholesterol remaining in the plasma after precipitation of the apoB-containing lipoproteins with heparin-calcium (Ciba-Corning, OH) or dextran-sulfate (Bayer Corp. Tarrytown, NY). Plasma levels of apolipoproteins were quantified by Western blot analysis followed by densitometric scanning as described (29;32).

**Western Blot Analysis**—Mouse liver proteins purified as previously described (34) were electrophoresed on 4-12% Bis-Tris acrylamide gels (Invitrogen), transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) and incubated with primary antibodies against human LDLr (RDI, Flanders, NJ), mouse SR-BI (Novus Biologicals, Littleton, CO) and mouse anti-LRP (a generous gift from Dr. Dudley K. Strickland) (33). The secondary antibody was a donkey anti-rabbit or goat anti-mouse antibody conjugated to horseradish peroxidase (Amersham, Piscataway, NJ). Proteins were detected by chemiluminescence (33).

**Fast Protein Liquid Chromatography and Native Agarose Gel Electrophoresis**—Plasma lipoproteins were analyzed by native agarose gel electrophoresis and FPLC (50ul of plasma pooled from 4 mice) as previously described (29;32;33).

**Bile Cannulations and Bile Assays**—Mice fed different diets for 10-12 weeks were weighed, fasted for 4-6 hours and anesthetized with IP avertin (1.25%; 0.015-0.017 ml/gram body weight) at mid-dark cycle. The cystic duct, exposed by an upper midline abdominal incision, was clamped and the common bile duct was cannulated with a PE-10 polyethylene catheter with an inside diameter of 0.28 mm and an outside diameter 0.61 mm (Becton Dickinson Primary Care Diagnostics, Sparks, MD) superior to the sphincter of Oddi but inferior to the entrance of the cystic duct. Bile was collected for 45 minutes by gravity. Animals were maintained at 37°C with a heat lamp. Total bile sterols were measured by both GC (Model GC2010, Shimadzu, Columbia, MD) as
described below and by using the Sigma Cholesterol Assay kit (Sigma, St. Louis, MO). Bile acids were assayed with the Sigma Bile Acid Assay Kit (Sigma, St. Louis, MO). Bile phospholipid content was assayed with the WAKO Phospholipid Assay Kit (Wako, Richmond, VA).

**Analysis of Sterol Composition**—Aliquots of plasma and bile for HPLC analysis were extracted in chloroform:methanol 2:1 (v:v) and separated by addition of water (35). Samples were hydrolyzed with 1M ethanolic KOH at 80°C for 1 hr and extracted twice with hexane. Sterols were converted to their benzoate derivatives with benzoyl chloride reagent, re-extracted with dichloromethane, dissolved in acetonitrile-dichloromethane 2:1 (v:v) and injected into a reverse phase column Ultrasphere 5µ C18 150 x 4.6 mm (Beckman Coulter, Fullerton, CA) according to the method of Kasama (36). The eluting solvent was acetonitrile:acetic acid (100:0.2) at a flow rate of 0.5 ml/min. The instrument was a Waters 600 controller, 717 autosampler, and 486 tunable absorbance detector with column oven (Waters, Milford, MA). Peaks were processed using the Waters Millenium³², version 3.2 software, using sterol standards obtained from Sigma-Aldrich (St. Louis, MO).

**Cholesterol Absorption**—Male mice (4-8 months of age) maintained on the RC diet were used for cholesterol absorption studies by the plasma dual-isotope ratio method (37;38).

**In Vivo Cholesterol Synthesis**—The rates of in vivo cholesterol synthesis in the liver and intestine during mid-dark cycle were measured after intraperitoneal injection of 10 mCi of [³⁵S] water (New England Nuclear Corp.) as described (39;40).

**Hepatic Sterol and Fecal Neutral Sterol Content**—The concentration of total neutral sterols in liver and feces was measured by GC (Shimadzu) as previously described (38) with the following minor modifications. Approximately 100 mg (wet weight) of liver and 50 mg (dry weight) of ground stool was homogenized in 1 ml H₂O. Sterols were extracted by adding 21 mls of chloroform-methanol (2:1, vol/vol) and 4.5 mls H₂O (35;41). Aliquots of the organic extract were saponified in ethanolic-KOH, sterols were extracted in hexane and an aliquot of the hexane extract was dried and dissolved in ethyl acetate. The concentration of total neutral sterols in ethyl acetate was then
measured by GC. Fecal excretion of cholesterol and its breakdown products was calculated by adding coprostanol, cholestanol and cholesterol measured in feces by GC. In parallel studies, dried aliquots of the Folch extract were dissolved in isopropanol for analysis of hepatic and fecal total neutral sterols using an enzymatic assay as described (41).

Analysis of Aortic Lesions—The heart and attached section of ascending aorta were dissected en bloc and prepared as previously described (30). Three mm sections of the aortic root and ascending aorta were stained with oil-red-O for neutral lipids and hematoxylin for nucleic tissue. Five sections per animal were evaluated to determine the mean cross-sectional area of lesions for each animal (30).

Statistical Analysis—All data are expressed as means +/- SEM. Statistically significant differences between control and transgenic mice were assessed by Student’s t-test and defined as a two-tailed probability of less than 0.05. Non-parametric data was analyzed by the Mann-Whitney test (Instat Software, Graphpad, Inc., San Diego, CA).

RESULTS

Characterization of Transgenic Mice with Liver-Specific hABCG5/G8 Overexpression on a Low Sterol Diet

Liver-Specific Overexpression of Human ABCG5/G8 Does Not Alter Hepatobiliary Sterol Secretion and Intestinal Cholesterol Absorption on a Low Sterol Diet—ABCG5 and ABCG8 expressed in liver and intestine have important roles in biliary and intestinal sterol transport (10-12;23) but the independent contribution of hepatic ABCG5/ABCG8 has not yet been investigated. We generated a transgenic mouse line (Tg) with 63 copies of the human ABCG5 and ABCG8 genes under control of their native promoter by injecting a BAC construct containing the entire hABCG5/G8 locus (28) into C57Bl/6 mice. Northern analysis using probes that detect both mouse and human ABCG5/G8 demonstrated that ABCG5 and ABCG8 mRNA was increased in the liver (~2-3-fold) but not small intestine of liver G5/G8-Tg mice compared to nontransgenic control mice (Fig. 1A; left panels). Northern analysis using human-specific probes (hG5 and hG8; Fig. 1A;
middle panels) confirmed the presence of human ABCG5 and ABCG8 transcripts in the liver, but not small intestine, of liver G5/G8-Tg mice. Human ABCG5/G8 overexpression did not alter mABCG5 or mABCG8 mRNA levels in the liver or intestine of liver G5/G8-Tg mice (Fig. 1A; right panels). These findings were further confirmed by real time RT-PCR using human- or mouse-specific primers (data not shown). The liver-specific pattern of hABCG5/G8 gene expression in liver G5/G8-Tg mice allowed us to investigate the contribution of liver ABCG5 and ABCG8 to sterol transport and atherosclerosis.

Table IA summarizes the effects of hepatic ABCG5/G8 overexpression on hepatic and intestinal sterol transport in C57Bl/6 mice fed a low sterol (regular chow) diet. Hepatic ABCG5/G8 overexpression did not significantly increase the hepatobiliary sterol concentrations (772.2+/−39.3 μM vs 830.2+/−76.9 μM) or secretion rates (2.36+/−0.15 vs 2.35+/−0.17 umol/hr/kgBW) of liver G5/G8-Tg mice over that of controls (Table IA). Biliary phospholipids (Tg-14.1+/−1.2 vs C-18.3+/−1.8 umol/hr/kg BW) as well as biliary bile acid secretion rates (Tg-123+/−13 vs C-153+/−14 umol/hr/kg BW) were also similar in liver G5/G8-Tg and control mice. On the RCD (0.025% cholesterol, 0.072% plant sterols) liver G5/G8-Tg and control C57Bl/6 mice ingested similar amount of sterols, approximately 1.10+/−0.20 mg of cholesterol (Table IA) and 3.21+/−0.22 mg of plant sterols daily. Intestinal cholesterol absorption, measured by the plasma dual-isotope method (37;38) was unchanged in liver G5/G8-Tg mice (n=6; 38.80%/+−3.43) compared to controls (n=17; 40.20%/+−2.00) (Table IA). As a result, the daily amount of cholesterol (in mg) available for intestinal absorption and fecal excretion (defined as dietary plus biliary cholesterol) in liver G5/G8-Tg (1.83+/−0.21 mg/d/30gm BW) and control (1.83+/−0.20 mg/d/30gm BW) mice as well as the actual mg quantities of absorbed cholesterol (defined as intestinal cholesterol x % cholesterol absorption) in liver G5/G8-Tg (0.71+/−0.21mg/d/30gm BW) and control (0.74+/−0.21mg/d/30gm BW) mice were similar (p>0.05; all) (Table IA). Fecal neutral sterol excretion, which on a low sterol diet (0.025% cholesterol, 0.072% plant sterols) consists mostly of dietary plant sterols, was unchanged in liver G5/G8-Tg (n=9; 2.96+/−0.14 mg fecal sterol/day) vs control (n=11; 2.91+/−0.27-GC mg fecal sterol/day) mice. Similarly, no difference in the daily fecal cholesterol excretion was evident between Tg (n=9; n=6, 1.17+/−0.06 mg
Contribution of hepatic ABCG5/G8 overexpression to sterol transport and atherosclerosis

fecal cholesterol/day) and control (n=11; 1.08+/-0.21-GC mg fecal cholesterol/day) mice. Not surprisingly, no differences in either the hepatic cholesterol concentrations (Table IA) or plasma lipids (Fig. 1B and C) or plant sterol concentrations (Fig. 1D) were evident in liver G5/G8-Tg and control mice.

Thus, on the low sterol diet the increased levels of human ABCG5/G8 expression did not alter the biliary sterol excretion or intestinal cholesterol absorption already mediated by the endogenous mouse ABCG5 and ABCG8 transporters in our liver G5/G8-Tg mice.

Characterization of Transgenic Mice with Liver-Specific hABCG5/G8 Overexpression on a 4% Cholesterol Diet

Increased Dietary Cholesterol Enhances Expression of ABCG5/G8 and Raises Hepatic and Plasma Cholesterol Levels in both Liver G5/G8-Tg and Control Mice—We first analyzed the general effects of the cholesterol-rich diet on both control and liver G5/G8-Tg mice. Northern analysis using probes that recognize both mouse and human ABCG5/G8 (Fig. 2A) demonstrated that, compared to the RCD, the 4%C diet increased expression of ABCG5 and ABCG8 in the liver and intestine of both liver G5/G8-Tg and control mice (by approximately 2 fold). Further studies using mouse- and human-specific probes showed that the cholesterol-rich diet enhanced hepatic expression of mouse ABCG5 and ABCG8 in both Tg and control mice and of human ABCG5/G8 in Tg mice. As on the RCD, human ABCG5/G8 mRNA was increased in liver but not intestine of liver G5/G8-Tg mice compared to control mice (Fig. 2A).

Compared to the low sterol diet (RCD), the 4%C diet raised the fasting plasma levels of TC, TG, FC and CE (~1.2-fold; p<0.0001; all, compare lipids in Fig. 1B vs. Fig. 2B) and increased hepatic cholesterol content (~ 3 fold; p<0.0001; Table IA vs IB) of both liver G5/G8-Tg and control study groups. Increased dietary cholesterol also significantly raised the biliary sterol secretion rate of control (1.5-fold) and liver G5/G8-Tg (2.9-fold) mice compared to similar mice on a RCD (Table IA vs IB). Moreover, on the 4%C diet, the increased absorption of dietary cholesterol and expanded pool of plasma cholesterol was associated with significant reductions (~70-90%) in the plasma concentrations of campesterol and sitosterol and the ratio of campesterol/cholesterol and
sitosterol/cholesterol in both Tg and control mice (Fig. 2C) compared to those present in similar mice fed the low sterol diet (Fig. 1D). These findings are consistent with previous studies showing that increased dietary cholesterol leads to significant accumulation of cholesterol in the plasma and liver of mice despite compensatory mechanisms such as downregulation of hepatic cholesterol synthesis and enhanced biliary sterol secretion (42-44).

Hepatic Overexpression of hABCG5/G8 Enhances Biliary Sterol Secretion in Liver G5/G8 Transgenic Mice on the 4%C Diet—As discussed above, on the 4%C diet, not only is ABCG5/G8 gene expression enhanced but in addition, hepatic cholesterol concentrations and hence, the amount of cholesterol substrate available for transport by liver ABCG5/G8 is increased. These two factors likely contributed to our findings, that in contrast to the RCD, overexpression of ABCG5/G8 in liver significantly increased biliary sterol concentrations by 2.1-fold (2123.74+/−99.00 μM vs 993.70+/−17.30 μM) and secretion rates by 1.9-fold (6.73+/−0.31 vs 3.63+/−0.07 umol/hr/kg BW in liver G5/G8-Tg mice compared to nontransgenic controls (Table IB) on the 4%C diet. Hepatic ABCG5/G8 overexpression also increased the campesterol/cholesterol ratio in bile (liver G5/G8-Tg =0.048+/−0.003; Control =0.037+/− 0.001; p=0.015). No differences in biliary phospholipid (25.8+/−1.2 vs 26.9+/−1.2 umol/hr/kg BW) or biliary bile acid (183+/−9 vs 166+/−11 umol/hr/kg BW) secretion rates were evident between control and Tg mice, respectively.

On the 4%C diet, compared to the low sterol diet, the percent intestinal cholesterol absorption was reduced in both control (n=13; 25.82+/−1.49%) and liver G5/G8-Tg (n=13; 26.43+/1.72%) mice (Table IB). This finding is consistent with previous reports demonstrating that when the amount of dietary cholesterol is increased fractional intestinal cholesterol absorption is decreased (45). As on the RCD no significant difference in intestinal cholesterol absorption was evident between the two mouse study groups. These combined findings demonstrate that selective hepatic overexpression of ABCG5/G8 enhances biliary sterol secretion but does not alter intestinal cholesterol absorption in mice.

Table IB summarizes the hepatic and intestinal sterol transport in control and liver
G5/G8-Tg mice. On the 4%C diet (3.9% cholesterol and 0.065% plant sterols) each 30 gm mouse ingests approximately 170.73+/−3.37 mg of dietary cholesterol (Table IB) and 2.60+/−0.15 mg of plant sterols daily. Compared to dietary cholesterol, the contribution of biliary cholesterol (Tg-2.08+/−0.09 vs controls-1.13+/−0.02 mg/d/30gm BW; Table IB) to the total daily load of intestinal cholesterol available for absorption from dietary plus biliary sources (Tg-172.81+/−3.38 and controls-171.86+/−3.37 mg/d/30gm BW; Table IB) is very small (less than 1% for both control and liver G5/G8-Tg mice) on the 4%C diet. Hepatic overexpression of ABCG5/G8 doubled the daily hepatobiliary sterol secretion rate (controls-1.13+/−0.02 vs Tg-2.08+/−0.09 mg/d/30gm BW; p<0.002; Table IB) increasing the amount of biliary sterol secreted daily by 0.95+/−0.27 mg/d/30gm BW in Tg vs control mice. However, the extra hepatobiliary sterols secreted by liver G5/G8-Tg mice had a minimal impact (<0.8% increase) on the total amount of intestinal cholesterol available for absorption and fecal excretion (Table IB). Furthermore, in the absence of changes in the fractional intestinal cholesterol absorption (liver G5/G8-Tg; 26.43+/−1.72% and controls; 25.82+/−1.49%), the daily mg amount of cholesterol absorbed from the gut and transported via chylomicrons back to the liver in liver G5/G8-Tg (45.67+/−3.46 mg/d/30gm BW) vs control (44.37+/−3.43 mg/d/30gm BW) mice was, in fact, greater (by 1.30 mg/d) in Tg mice (Table IB), although these differences did not reach statistical significance (p>0.05). Hence, the increase biliary sterols secreted by liver G5/G8-Tg vs control mice were reabsorbed by the intestine rather than excreted in the feces. Total neutral fecal sterol excretion (Tg, n=6; 108.68+/−3.98 mg/d vs controls, n=6; 108.74+/−3.63 mg/d) and fecal cholesterol excretion (Tg, n=6; 105.42+/−3.86 mg/d vs controls, n=6; 105.48+/−3.52 mg/d) were similar (p>0.05; all) in liver G5/G8-Tg vs control mice on the 4%C diet.

Since feeding a cholesterol-rich diet can alter the fractional cholesterol absorption measured by the dual isotope method we also determined intestinal cholesterol absorption using the cholesterol balance approach (45). The mg amounts of cholesterol absorbed daily were calculated by subtracting the mg of cholesterol excreted in the feces each day from the total amount of cholesterol present daily in the intestine of Tg and control mice (Table IB; Intestinal Cholesterol Diet + Bile). Although for both control and Tg mice the calculated amount of cholesterol absorbed daily was higher using the
cholesterol balance method (Tg=67.39±5.12 mg/d and C=66.38±4.89 mg/d) than using the fractional cholesterol absorption data (Tg=45.67±3.46 mg/d vs control 44.37±3.43 mg/d), intestinal cholesterol absorption was not decreased in liver G5/G8-Tg vs control mice by either approach. Consistent with the fractional intestinal cholesterol absorption findings, the cholesterol balance approach revealed that the increase in daily biliary sterol secretion in Tg mice (~0.95 mg/d) was offset by a ~1.02 mg/d increase in the daily mg amount of cholesterol absorbed from the gut and transported via chylomicrons back to the liver in liver G5/G8-Tg. Thus, although hepatic overexpression of ABCG5/G8 enhanced hepatobiliary sterol secretion (by 1.9 fold) on the 4%C diet, it did not alter the cholesterol balance across the liver nor increase removal of body cholesterol from liver G5/G8-Tg vs control mice. Consequently, the increased biliary sterol secretion observed in liver G5/G8-Tg mice was not associated with reductions in the hepatic cholesterol content (Table IB) or compensatory alterations in liver expression of the LDL receptor, ABCA1, ABCG1, SR-BI, HMGCoA reductase and 7-alpha hydroxylase compared to control mice (data not shown).

Hepatic Overexpression of hABCG5/G8 Alters Plant Sterol Concentrations in Plasma on the 4%C Diet—Analysis of the plasma lipid profile revealed no differences between transgenic and control mice on the 4%C diet (Fig. 2B). Hepatic overexpression of ABCG5/G8 did, however, significantly reduce the plasma campesterol levels (by ~35%) and campesterol/cholesterol ratio (in liver G5/G8-Tg mice compared to controls) (Fig. 2C) indicating that overexpression of ABCG5 and ABCG8 only in liver is sufficient to decrease the accumulation of plant sterols in plasma.

Hepatic Overexpression Of hABCG5/G8 Does Not Alter Aortic Atherosclerosis in Transgenic C57Bl/6 Mice on a HF-HC Diet—To investigate the effect of ABCG5 and ABCG8 overexpression on aortic lesion formation in C57Bl/6 mice, we placed liver G5/G8-Tg and control mice on a high cholesterol, cholate-containing (HF-HC) diet for 12 weeks (30;31). This cholate-enriched diet is necessary to induce lesion formation in C57Bl/6 mice. As expected, the plasma TC, PL, CE, and FC plasma levels were increased in all 3 mouse study groups (data not shown). However, no significant differences in the plasma lipid profiles of liver G5/G8-Tg compared to control nontransgenic mice were evident after 12 weeks of the HCC diet (data not shown).
addition, the mean aortic lesion areas in control (2146+/−330 mm²) and liver G5/G8-Tg (2509+/−846 mm²) mice were similar. Since the cholate-enriched diet markedly alters sterol absorption (46) and pathways necessary for maintaining hepatic cholesterol homeostasis (47;48) this diet may mask potential benefits resulting from the overexpression of ABCG5 and ABCG8. Consistent with this concept, we found no differences in biliary sterol flow between liver G5/G8-Tg and control mice on the HF-HC diet [controls (n=14), 22.5+/−5.3 um/hr/kg BW; Tg (n=8), 23.4+/−5.5 um/hr/kg BW, p=0.91].

**Hepatic Overexpression of hABCG5/G8 Enhances Hepatobiliary Sterol Excretion, but Does Not Alter the Plasma Lipoproteins or Aortic Atherosclerosis in ApoE-KO Mice Fed a RCD**

*Generation of ApoE x hABCG5/G8-Tg Mice*—We then investigated the effects of hepatic ABCG5 and ABCG8 overexpression on sterol transport and aortic atherosclerosis in a mouse model that does not require high levels of dietary cholate to induce lesion formation; apoE-KO mice were crossed with liver G5/G8-Tg mice to generate liver G5/G8-Tg x E-KO mice. As observed in Tg mice on a C57Bl/6 background (Fig. 1A), both human transgenes were primarily expressed in the liver but not the small intestine of liver G5/G8-Tg x E-KO mice (Fig. 3A) and expression of mouse ABCG5 and ABCG8 was similar between liver G5/G8-Tg x E-KO and E-KO mice in both the liver and the intestine (Fig. 3A).

The plasma cholesterol, PL, FC, CE and non-HDL cholesterol (Fig. 3B vs Fig. 1B) as well as the hepatic cholesterol content (Table IC vs. Table IA) of E-KO and liver G5/G8-Tg x E-KO mice were increased compared to C57Bl/6 and Tg mice on a RCD. Furthermore, both E-KO and liver G5/G8-Tg x E-KO Tg mice had markedly increased biliary sterol secretion rates (3-4 fold) compared to the analogous C57Bl/6 mice (Table IC vs Table IA).

**Hepatic Overexpression of hABCG5/G8 in ApoE-KO Mice Enhances Biliary Sterol Secretion but Does Not Alter the Plasma Lipoproteins or Aortic Atherosclerosis in Liver G5/G8 ApoE-KO x Tg Mice**—Overexpression of ABCG5 and ABCG8 in liver increased the biliary sterol concentration (1689.47+/−38.09 uM vs 2602.87+/−63.58 uM; ~1.5-fold;
p<0.0005) and secretion rates (6.12+/-0.20 vs 8.84+/-0.41 umol/hr/kgBW; ~1.4-fold; p=0.03) in liver G5/G8-Tg x E-KO compared to E-KO mice (Table IC). As on the 4%C diet, enhanced hepatobiliary sterol secretion in liver G5/G8-Tg x E-KO vs E-KO mice occurred in the context of increased hepatic cholesterol content (Table IC), compared to liver G5/G8-Tg and C57Bl/6 mice on RCD (Table IA), which increases the amount of cholesterol substrate available for ABCG5/G8 transport. As before, the biliary phospholipid and bile acid secretion rates were similar between E-KO and liver G5/G8-Tg x E-KO mice (data not shown). Fractional intestinal cholesterol absorption was not decreased in liver G5/G8-Tg x E-KO vs control E-KO mice; in fact, a trend for increased cholesterol absorption that did not reach statistical significance (p<0.09) was evident in liver G5/G8-Tg (n=12, 75.87 +/- 2.23%) vs control E-KO (n=22; 64.73 +/- 3.53%) mice on the RCD (Table IIC). These data again demonstrate that selective hepatic overexpression of ABCG5/G8 enhances biliary sterol but not intestinal sterol transport.

Table IC summarizes the hepatic and intestinal transport of sterols in E-KO and liver G5/G8-Tg x E-KO mice. On the RCD, both groups of mice ingest 1.20 +/- 0.05 mg of cholesterol per day and secrete 1.90 +/- 0.06 mg/d (E-KO) and 2.74 +/- 0.13 mg/d (liver G5/G8-Tg x E-KO) of sterols in the bile daily. In these mice the contribution of biliary cholesterol to the daily load of intestinal cholesterol available for absorption from dietary plus hepatobiliary sources (E-KO; 3.10 +/- 0.08 mg/d and liver G5/G8-Tg x E-KO; 3.94 +/- 0.14 mg/d) is significant (~61%-E-KO and 69%-liver G5/G8-Tg x E-KO). Thus, the extra hepatobiliary sterol secreted by liver G5/G8-Tg x E-KO mice vs E-KO (0.84 +/- 0.31 mg/d) represents an increase of approximately 27% in the daily amount of intestinal cholesterol available for absorption and fecal excretion. Since intestinal cholesterol is greater in liver G5/G8-Tg x E-KO mice than E-KO mice and the fractional intestinal cholesterol absorption in liver G5/G8-Tg x E-KO (75.87 +/- 2.23%) was not decreased compared to nontransgenic E-KO (64.73 +/- 3.53%) mice, the daily amount of cholesterol absorbed from the gut and transported to the liver is anticipated to be increased in liver G5/G8-Tg x E-KO mice. We found an increase (approximately 0.99 +/- 0.18 mg/d) in the calculated daily intestinal cholesterol absorption (Table IC) liver G5/G8-Tg x E-KO (2.99 +/- 0.14 vs E-KO) vs. E-KO (2.00 +/- 0.11 mg/d/30gm BW) mice. Thus, the increase hepatic cholesterol output via the biliary system (~0.84 mg/d) in liver G5/G8-Tg x E-KO
mice was offset by greater input of cholesterol (~0.99 mg/d) to the liver resulting from absorption and transport of intestinal cholesterol via chylomicrons. Similar results were obtained when the sterol balance approach was used to calculate the difference in mg of intestinal cholesterol absorbed daily between E-KO (1.91 +/- 0.2) and liver G5/G8-Tg x E-KO mice (2.91 +/- 0.16) using measured fecal cholesterol excretion data (see below). The daily fecal neutral sterol excretion (E-KO, n=6; 2.40 +/- 0.35 mg/d and liver G5/G8-Tg x E-KO, n=6; 2.53 +/- 0.13 mg/d) and fecal cholesterol excretion (E-KO, n=6; 1.19 +/- 0.18 mg/d and liver G5/G8-Tg, n=6; 1.03 +/- 0.08) were similar in apoE-KO and liver G5/G8-Tg x E-KO mice. These combined data indicate that, despite a 1.5-fold increase in biliary cholesterol secretion and a significant 27% increase in intestinal cholesterol, in the absence of changes in fractional intestinal cholesterol absorption hepatic overexpression of ABCG5/G8 did not significantly alter the cholesterol balance across the liver nor increase the daily excretion of fecal cholesterol of liver of E-KO x liver G5/G8-Tg vs control E-KO mice.

Consistently, the increased biliary sterol secretion in liver G5/G8-Tg x E-KO mice was not associated with changes in either hepatic cholesterol concentrations (Table IIIC), liver cholesterol synthesis (E-KO, n=4; 347.57 +/- 82.35 vs liver G5/G8 Tg x E-KO, n=4; 308.94 +/- 79.30 nmole/hr/g; p>0.05), or changes in liver expression of the LDL receptor, ABCA1, ABCG1, SR-BI, HMGCoA reductase and 7-alpha hydroxylase compared to E-KO mice (data not shown). Furthermore, no significant differences in the mean fasting plasma concentrations of TC, TG, PL, CE, HDL-C, apoA-I, and apoB or in the plasma lipoproteins (Fig. 3B and 3C) were evident between E-KO and liver G5/G8-Tg x E-KO mice. Hepatic overexpression of ABCG5/G8 did, however, reduce the campesterol levels (by ~25%) and campesterol/cholesterol ratio (by ~20%) in plasma of liver G5/G8-Tg x E-KO compared to E-KO mice (Fig. 3D).

To evaluate whether the increased biliary sterol secretion induced by hepatic ABCG5/G8 overexpression altered atherosclerosis, we analyzed the proximal aortas of 3 and 4 month old female and 4-month old male liver G5/G8-Tg x E-KO and apoE-KO mice fed a RCD (Fig. 3E). As anticipated by the absence of changes in the plasma lipid profile, no differences in proximal aortic lesions were detected between the aortas of 3 month old and 4 month old female or between aortas of 4 month old male liver G5/G8-
Tg x E-KO and E-KO mice.

**Hepatic Overexpression of hABCG5/G8 Enhances Hepatobiliary Sterol Excretion, but Does Not Alter the Plasma Lipoproteins or Alter Aortic Atherosclerosis In LDLr-KO Mice Fed a Western Diet**

**Generation of LDLr-KO x hABCG5/G8-Tg Mice**—We also investigated the effects of hepatic ABCG5 and ABCG8 overexpression on sterol transport and aortic atherosclerosis in a second atherosusceptible mouse model by crossing LDLr-KO mice with liver G5/G8-Tg mice. As in C57Bl/6 and E-KO mice, both human transgenes were primarily expressed in the liver but not small intestine of liver G5/G8 LDLr-KO x Tg mice (Fig. 4A) and expression of mouse ABCG5 and ABCG8 was similar between both study groups in both liver and intestine (Fig 4A).

On the Western diet the plasma cholesterol levels (Fig. 4B vs Fig. 1B) and hepatic cholesterol content (Table ID vs. Table IA) of LDLr-KO and liver G5/G8 LDLr-KO x Tg mice were increased compared to C57Bl/6 mice on a RCD. Both LDLr-KO and liver G5/G8 LDLr-KO x Tg mice had markedly increased biliary sterol (3-6 fold) secretion rates compared to the analogous C57Bl/6 mice (Table ID vs Table IA).

**Hepatic Overexpression of hABCG5/G8 in LDLr-KO Mice Enhances Biliary Sterol Secretion but not Aortic Atherosclerosis in Liver G5/G8 Tg x LDLr-KO Mice**—Overexpression of ABCG5 and ABCG8 in liver increased the biliary sterol secretion rates in liver G5/G8-Tg x LDLr-KO mice compared to LDLr-KO mice (13.75+/-1.18 vs. 7.94+/-0.94 umol/hr/kgBW;1.7-fold, p<0.002) (Table ID). As before, the biliary phospholipid and bile acid secretion rates were similar between LDLr-KO and LDLr-KO x Tg mice (data not shown) and no significant difference (p=0.09; all) in fractional intestinal cholesterol absorption was evident between LDLr-KO (n=5, 46.39+/-2.09%) and liver G5/G8 Tg x LDLr-KO mice (n=8, 57.7+/-3.53%) (Table ID).

Table ID summarizes the hepatic and intestinal sterol transport in LDLr-KO and liver G5/G8-Tg x LDLr-KO mice. On the Western diet (0.2% cholesterol, <0.003% plant sterols) both groups of mice ingest approximately 7.96+/-0.426 mg of cholesterol per (Table IID). As with E-KO mice, the biliary cholesterol (LDLr-KO; 2.46+/-0.29 mg/d and liver G5/G8-Tg x LDLr-KO; 4.26+/-0.37 mg/d) significantly contributed to the daily load of
Contribution of hepatic ABCG5/G8 overexpression to sterol transport and atherosclerosis

intestinal cholesterol available for absorption and fecal excretion from dietary plus biliary sources in LDLr-KO (10.42+/-0.52 mg/d; ~23%) and liver G5/G8-Tg x LDLr-KO (12.22+/-0.56 mg/d; ~35%). The extra hepatobiliary sterols secreted by liver G5/G8-Tg x LDLr-KO mice (4.26+/-0.37 mg/d) vs LDLr-KO (2.46+/-0.29 mg/d; p<0.006) mice (1.80+/-0.52 mg/d) represents approximately an 18% increase in the daily load of intestinal cholesterol available for absorption and fecal excretion in LDLr-KO mice. Since the fractional intestinal cholesterol absorption was not decreased in liver G5/G8-Tg x LDLr-KO (57.70+/-3.53%) and LDLr-KO (46.39+/-2.09%; p=0.09) mice, the daily amount of cholesterol absorbed from the gut and transported to the liver was increased (by ~2.22+/-0.81 mg/d) in liver G5/G8-Tg x LDLr-KO (7.05+/-0.61 mg/d) vs LDLr-KO mice (4.83+/-0.52 mg/d). Thus, the increase hepatic cholesterol output (via bile) in liver G5/G8-Tg x LDLr-KO mice (~1.79+/-0.52 mg/d) was offset by increased input of cholesterol to the liver resulting from absorption and transport of intestinal cholesterol via chylomicrons (2.22+/-0.81 mg/d). Fecal cholesterol excretion (liver G5/G8-Tg x LDLr-KO, n=6; 4.06+/-0.44 mg/d) and LDLr-KO, n=5; 4.39+/-0.61 mg/d) was not enhanced in liver G5/G8-Tg x LDLr-KO vs LDLr-KO mice.

Although for both LDLr-KO and liver G5/G8-Tg x LDLr-KO mice fed a Western diet the calculated amount of cholesterol absorbed daily was higher using the cholesterol balance method (Tg x LDLr-KO; 8.16+/-0.71 and LDLr-KO 6.03+/-0.80 mg/d) than using the fractional cholesterol absorption data (Tg x LDLr_KO; 7.05+/-0.61 vs LDLr-KO; 4.83+/-0.52 mg/d/30gm BW both methods yielded similar differences in the mg of intestinal cholesterol absorbed daily in liver G5/G8-Tg x LDLr-KO mice vs LDLr-KO mice.

These combined data indicate that, despite a 1.7-fold increase in biliary cholesterol secretion and an 18% increase in intestinal cholesterol, in the absence of changes in fractional intestinal cholesterol absorption hepatic overexpression of ABCG5/G8 did not significantly alter the cholesterol balance across the liver nor increase the daily excretion of fecal cholesterol liver G5/G8-Tg x LDLr-KO vs control LDLr-KO mice.

Consistently, the increased biliary sterol excretion in liver G5/G8-Tg x LDLr-KO mice was not associated with either decreased hepatic cholesterol concentrations (Table IIC),
increased hepatic cholesterol synthesis (LDLr-KO, n=4; 555.27+/−64.24 vs liver G5/G8-Tg x LDLr-KO, n=4; 338.16+/−92.54 nmol/hr/g; p>0.05) or compensatory changes in liver expression of the LDL receptor, ABCA1, ABCG1, SR-BI, HMGCoA reductase and 7-alpha hydroxylase compared to LDLr-KO mice (data not shown). Analysis of the plasma lipid profile revealed no significant differences in the mean fasting plasma concentrations of TC, TG, PL, CE, HDL-C, apoA-I, apoE and apoB on either RCD (data not shown) or Western diet (Fig. 4B and 4C) between LDLr-KO and liver G5/G8-Tg x LDLr-KO mice. Although on the Western diet no differences in the plasma concentrations of campesterol, sitosterol as well as plant sterol/cholesterol ratios were evident (data not shown), hepatic overexpression of ABCG5/G8 reduced the campesterol concentrations (by 30%) as well as the campesterol/cholesterol ratio (by 31%) in plasma of liver G5/G8-Tg x LDLr-KO compared to LDLr-KO mice on the RCD (Fig. 4D).

To evaluate whether the increased biliary sterol excretion induced by ABCG5/G8 overexpression altered atherosclerosis, we analyzed the proximal aortas of 10- and 13-week-old male and female LDLr-KO and liver G5/G8-Tg x LDLr-KO mice fed a Western diet (Fig. 4E). No difference in proximal aortic lesions were evident in either female or male liver G5/G8-Tg x LDLr-KO compared to LDLr-KO mice placed on a Western diet for either 10 or 13 weeks (Fig. 4E).

**DISCUSSION**

While previous work has demonstrated that ABCG5 and ABCG8 are ABC half-transporters expressed primarily in liver and intestine that function to transport sterols (10-12;23;24) the individual roles of hepatic vs intestinal ABCG5/G8 in sterol transport and atherosclerosis have not yet been delineated. Here we report the generation of transgenic mice that selectively overexpress human ABCG5 and ABCG8 in liver of C57BL/6, apoE-KO, and LDLr-KO mice under control of their native promoters. Using this transgenic mouse line, we were able to investigate the specific contributions of hepatic hABCG5/G8 to sterol transport and atherosclerosis under different dietary...
regimens. Hepatic ABCG5/G8 overexpression increased biliary sterol concentration and secretion and limited accumulation of plant sterols in plasma under conditions of high dietary or hepatic sterol load. Overexpression of ABCG5/G8 did not, however, affect intestinal cholesterol absorption, hepatic cholesterol concentrations, fecal sterol excretion or plasma cholesterol and lipoprotein levels under any conditions. Consequently, hepatic ABCG5/G8 overexpression did not protect against the development of aortic lesions in three independent mouse models of atherosclerosis.

Several lines of evidence demonstrate that the hABCG5 and hABCG8 transgenes were overexpressed and functional in liver. ABCG5/G8 mRNA levels were increased by approximately 2-3 fold in liver of Tg mice in all study groups, compared to control nontransgenic mice. Secretion of sterols into bile was increased in liver G5/G8-Tg mice (compared to control nontransgenic mice) on the 4%C diet, for liver G5/G8-Tg x E-KO mice on a RCD, and for liver G5/G8-Tg x LDLR-KO mice on a Western diet. Each of these conditions imposed a significant sterol load upon the mouse liver. Hepatic overexpression of ABCG5/G8 also functioned to decrease plant sterol concentrations and plant sterol/cholesterol ratios in plasma of Tg mice on these diets compared to control nontransgenic mice in the same study group. Interestingly, when the hepatic sterol load was normal (C57Bl/6 mice on a RCD), the biliary sterol secretion rates for liver G5/G8-Tg and nontransgenic control mice were similar. These data indicate efficient processing of normal quantities of hepatic sterols by the endogenous mouse transporters and suggest that in addition to changes in ABCG5/G8 gene expression, the amount of hepatic sterols available for transport may be important for ABCG5/G8 function. In all studies, control and liver G5/G8-Tg mice had similar biliary bile acid and phospholipid secretion rates, indicating that hepatic ABCG5/G8 is not involved in transport of these lipids. Altogether, these studies demonstrate that liver ABCG5/G8 function to decrease excess levels of hepatic cholesterol and plant sterols, but not bile acids or phospholipids.

We note that the increase in biliary sterol concentration due to hepatic ABCG5/G8 overexpression in our transgenic mice (1.5-2 fold) was less than that observed for the human ABCG5/G8 transgenic mice generated by Yu et al. (5-fold) (24), which express hABCG5/G8 in liver and intestine. These differences are likely due to different
ABCG5/G8 expression levels resulting from separate integration sites of the transgenes into the genome in these two independently generated ABCG5/G8 transgenic mouse lines. In addition, there are methodological differences between the two studies. We measured biliary sterol concentrations by the bile cannulation method which also permits calculation of daily hepatobiliary sterol secretion rates, whereas Yu et al. measured sterol concentrations in gall bladder bile. A major drawback to the latter method is that changes in gallbladder contractility and secretion or resorption of biliary components can lead to fluctuations in concentrations of gall bladder bile lipids. This is especially relevant for studies involving ABCG5/G8 since both half-transporters are expressed in the gall bladder (21). For these reasons, the bile cannulation method leads to more accurate and reproducible measurements, while also permitting direct assessment of daily biliary sterol secretion rates necessary for analysis of cholesterol balance.

Hepatic overexpression of ABCG5/G8 did not alter fractional intestinal cholesterol absorption measured by either the plasma dual isotope method or the cholesterol balance method (37;38) in any genetic mouse model on any dietary regimen. Previous studies have shown that fractional intestinal cholesterol absorption may be altered by changes in dietary cholesterol and biliary lipid secretion (45;49); increased intestinal cholesterol may dilute radiotracers used in the plasma dual isotope and fecal dual isotope methods as well as upregulate intestinal sterol transporters. For these reasons we also determined cholesterol absorption using the cholesterol balance approach (45). Using either method we demonstrate that hepatic ABCG5/8 overexpression does not reduce intestinal cholesterol absorption. The increase amount of intestinal cholesterol resulting from enhanced biliary cholesterol secretion in liver G5/G8-Tg mice could have resulted in upregulation of ABCG5/G8 gene expression in the small intestine; however, no changes in ABCG5/G8 mRNA or in fractional intestinal cholesterol absorption were observed. These combined data demonstrate that overexpression of ABCG5/G8 only in liver does not have a major impact on dietary cholesterol absorption. Using the fecal dual-isotope method, Yu et al. (24) measured a 50% reduction in fractional cholesterol absorption of mice overexpressing ABCG5/G8 in both liver and intestine. The decreased intestinal cholesterol uptake observed in the transgenic mice of Yu et al.
appears to be due to the intestinal overexpression of ABCG5/G8, as reported (24).

Increased hepatic expression of both half-transporters, significantly reduced the plasma concentrations of plant sterols, especially campesterol, and altered the plasma campesterol/cholesterol ratios in liver G5/G8-Tg mice. These findings suggest that there is a mechanism for selective removal of plant sterols from plasma by hepatic ABCG5/G8. Hepatic ABCG5/G8 could enhance the secretion of hepatic cholesterol and plant sterols into bile with equal affinity; depleted hepatic cholesterol pools may then be replenished by *de novo* cholesterol synthesis via HMGCoA reductase. Since this mechanism would replace lost cholesterol but not lost plant sterols, the plant sterol/cholesterol ratio in plasma would ultimately decrease. However, in our studies, the reduction in plasma plant sterols in liver G5/G8-Tg vs control mice persisted even when cholesterol levels in liver and plasma were high, and thus when *de novo* cholesterol synthesis was not expected to be increased. In fact, we found no evidence of increased cholesterol synthesis or changes in HMGCoA reductase gene expression in liver G5/G8-Tg vs control mice on any genetic background. Alternatively, any plant sterols absorbed by the intestine and transported to the liver may be preferentially secreted into the bile with each passage through the hepatobiliary tract by liver ABCG5/G8, thus selectively depleting plant sterols in plasma. The increased plant sterol/cholesterol ratios in bile of liver G5/G8-Tg compared to nontransgenic control mice support this concept. Our combined data indicate that hepatic ABCG5/G8 enhances secretion of sterols into bile, with an apparent preference for plant sterols and demonstrate that even in the absence of changes in intestinal ABCG5/G8 expression or fractional intestinal cholesterol absorption, enhanced biliary cholesterol secretion achieved by overexpressing ABCG5/G8 in liver, can protect against the accumulation of dietary plant sterols in plasma. Recent studies indicate that plant sterols in contrast to cholesterol, are unable to inhibit HMGCoA reductase activity in human monocyte-derived macrophages (17). Thus, preventing the accumulation of plant sterols in plasma may represent an important anti-atherogenic function for liver ABCG5/G8.

Previous studies have shown that in mice, approximately 80% of LDL is degraded in liver mostly via the LDL receptor pathway (50). Based on their known function in sterol transport, ABCG5 and ABCG8 are also anticipated to modulate lesion development.
Contribution of hepatic ABCG5/G8 overexpression to sterol transport and atherosclerosis

indirectly, by altering hepatic cholesterol stores, inducing compensatory changes in hepatic receptor and enzyme gene expression and hence, lowering pro-atherogenic plasma lipoproteins. Thus, our present knowledge of these two half-transporters would suggest that the anti-atherogenic potential of liver ABCG5/G8 may also depend on the ability of these half-transporters to induce a net loss of cholesterol from the liver. Depleted hepatic cholesterol pools could be replenished by reducing VLDL synthesis and/or enhancing expression of hepatic lipoprotein receptors which in turn, would lower the plasma levels of pro-atherogenic lipoproteins. In addition, compensatory increases in de novo hepatic cholesterol synthesis may occur; this latter compensatory response, which is robust in mice (51) would decrease the anticipated atheroprotective effects of ABCG5/G8 overexpression on the plasma lipoproteins.

In liver G5/G8-Tg mice a net loss of liver cholesterol would occur if ABCG5/G8 overexpression increased the output of cholesterol from the liver (via the biliary system) to a greater extent than the input of cholesterol to the liver (resulting from the absorption and transport of intestinal cholesterol via chylomicrons). Two different scenarios may lead to a net loss of liver cholesterol; overexpression of ABCG5/G8 could significantly decrease fractional intestinal cholesterol absorption (with or without changes in biliary cholesterol secretion); however, our data clearly demonstrate that a 2-3 fold increase in liver expression of ABCG5/G8 did not alter fractional intestinal cholesterol absorption in any of the animal models evaluated in our study. Alternatively, ABCG5/G8 overexpression could increase biliary cholesterol secretion to represent such a large fraction of the intestinal pool of cholesterol that even in the absence of changes in intestinal absorption, re-absorption of hepatobiliary cholesterol would be limited. In these two scenarios, the output of liver cholesterol via the bile would be greater than the input of cholesterol from the intestine in liver G5/G8-Tg mice thereby significantly altering hepatic cholesterol balance.

In the present study, hepatic overexpression of ABCG5/G8 did not alter hepatic cholesterol stores, hepatic cholesterol synthesis, hepatic expression of HMGCoA reductase, SR-BI or LDL receptor, nor alter the plasma lipid profile, including the plasma levels of pro-atherogenic plasma lipoproteins in C57Bl/6, apoE-KO or LDLr-KO mice. This combined data demonstrate that despite significantly enhancing (by 1.5-2 fold) the
output of liver cholesterol via the bile, increasing ABCG5/G8 expression only in liver and not intestine failed to induce a net loss of hepatic cholesterol in liver G5/G8-Tg mice and suggest that hepatic overexpression of ABCG5/G8 will not protect against the development of atherosclerotic lesions. Indeed this is what we observed in three mouse models: C57Bl/6 mice on a CCB/cholate diet; apoE-KO mice on a RCD, and LDLr-KO mice on a Western diet.

Analysis of changes in the cholesterol balance across the liver induced by hepatic ABCG5/G8 overexpression in our liver G5/G8-Tg mice provides insight into these findings. Figure 5 summarizes the sterol transport of E-KO vs liver G5/G8-Tg x E-KO mice (left panel) and of LDLr-KO vs liver G5/G8-Tg x LDLr-KO mice (right panel). Overexpression of ABCG5/G8 in liver significantly enhanced biliary cholesterol secretion in E-KO (by 1.4-fold) and LDLr-KO (by 1.7-fold) mice. Consequently, the amount of intestinal cholesterol available for absorption was also increased in liver G5/G8-Tg X E-KO vs E-KO mice (by 1.3-fold) and liver G5/G8-Tg x LDLr-KO vs LDLr-KO (by 1.2-fold) mice. However, since hepatic overexpression of ABCG5/G8 did not reduce the fractional intestinal cholesterol absorption in these two animal models, the daily amount of cholesterol absorbed from the intestine was also greater in liver G5/G8-Tg x E-KO vs E-KO (by 1.5-fold) and in liver G5/G8-Tg x LDLr-KO vs LDLr-KO (by 1.5-fold). Thus, the increased hepatic cholesterol output via the biliary system in both liver G5/G8-Tg x E-KO and liver G5/G8-Tg x LDLr-KO mice was offset by greater input of cholesterol to the liver resulting from absorption and transport of intestinal cholesterol via chylomicrons. Consequently, hepatic overexpression of ABCG5/G8 did not alter fecal cholesterol excretion in C57Bl/6, apoE-KO and LDLr-KO mice. No significant changes in bile acid fecal excretion are anticipated in these mice since biliary bile acid secretion was similar between control and liver G5/G8-Tg mice in all genetic backgrounds and previous studies have shown that bile acid pool size is not altered by ABCG5/G8 overexpression (24). Thus, hepatic overexpression of ABCG5/G8 only in liver did not alter hepatic cholesterol balance nor enhance the elimination of cholesterol from the body in liver G5/G8-Tg mice vs control mice.

Our combined results demonstrate that hepatic ABCG5/G8 overexpression functions to increase cholesterol and plant sterol secretion into bile and serves as an alternative
mechanism, independent of intestinal ABCG5/G8, to protect against the accumulation of potentially pro-atherogenic plant sterols in plasma. However, because increasing biliary cholesterol secretion expands the amount of intestinal cholesterol available for absorption, enhancing secretion of sterols in bile may not by itself, be sufficient to significantly alter hepatic cholesterol balance and therefore, protect against atherosclerosis. These studies do not rule out the possibility that in other species with less efficient intestinal cholesterol absorption, as in humans, greater hepatic overexpression of ABCG5/G8 might in fact, be atheroprotective. Our combined findings demonstrate that overexpression of ABCG5/G8 in liver profoundly alters hepatic but not intestinal sterol transport, identifying distinct roles for liver and intestinal ABCG5/G8 in modulating sterol metabolism.

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FIGURE LEGENDS

Fig. 1. Hepatic overexpression of human ABCG5/G8 in C67Bl/6 mice on a regular chow diet. Panel A. Northern blot analysis of total RNA isolated from liver and small intestine of control (C) and liver G5/G8-Tg (Tg) mice using probes that detect i) both mouse and human transporters (G5 and G8; left), ii) human transporters (hG5 and hG8; middle) and iii) mouse transporters (mG5 and mG8; right). Panel B. Fasting plasma lipids, lipoproteins and apolipoproteins (inset) in 3 month old female control and liver G5/G8-Tg mice (n=15-20, each group). Panel C. FPLC analysis and native agarose gel electrophoresis (inset) of pooled plasma (n=5, each group) from 3-month-old control and Tg female mice. Panel D. Plasma cholesterol, campesterol and sitosterol concentrations and plant sterol/cholesterol ratios in 3-month-old female control (white bars) and Tg (black bar) mice (n=4-6, each group).

Fig. 2. Hepatic overexpression of human ABCG5/G8 in C67Bl/6 mice on a 4%
cholesterol diet. Panel A. Northern blot analysis of total RNA isolated from liver and small intestine of control (C) and liver G5/G8-Tg (Tg) mice using probes that detect i) both mouse and human transporters (G5 and G8; left), ii) human transporters (hG5 and G8; middle) and iii) mouse transporters (mG5 and G8; right). For comparison, Northern blots hybridized with probes that detect both mouse and human transporters for control and Tg mice on both the RCD and 4% C are included (left). Panel B. Fasting plasma lipids, lipoproteins and apolipoproteins (inset) in 3-month-old female control and liver G5/G8-Tg mice (n=10-13, each group) on the 4% C diet. Panel C. Plasma cholesterol, campesterol and sitosterol concentrations and plant sterol/cholesterol ratios in 3 month old female control (white bars) and Tg (black bar) mice (n=4-6, each group) on the 4% C diet.

Fig. 3. Hepatic overexpression of human ABCG5/G8 in apoE-KO mice. Panel A. Northern blot analysis of total RNA isolated from liver and small intestine of E-KO and liver G5/G8-Tg x E-KO mice using probes that detect i) human transporters (hG5 and G8; top two panels) and ii) mouse transporters (mG5 and G8; panels 3 and 4 from top). Panel B. Fasting plasma lipids, lipoproteins and apolipoproteins (inset) of plasma from 3-month-old female E-KO and liver G5/G8-Tg x E-KO mice (n=8-16, each group). Panel C. FPLC analysis of pooled plasma (n=5, each group) from 3-month-old E-KO and liver G5/G8-Tg x E-KO mice. Panel D. Plasma cholesterol, campesterol and sitosterol concentrations and plant sterol/cholesterol ratios in 3-month-old female E-KO (white bars) and liver G5/G8-Tg x E-KO (black bar) mice (n=4-6, each group). Panel E. Mean proximal aortic lesion area in 3-month-old female, 4-month-old female and 4-month-old male E-KO and liver G5/G8-Tg x E-KO mice on a RCD.

Fig. 4. Hepatic overexpression of human ABCG5/G8 in LDLr-KO mice. Panel A. Northern blot analysis of total RNA isolated from liver and small intestine of LDLr-KO (L-KO) and liver G5/G8-Tg x LDLr-KO (L-KO x Tg) mice using probes that detect i) human transporters (hG5 and G8; top two panels) and ii) mouse transporters (mG5 and G8; panels 3 and 4 from top). Panel B and C. Fasting plasma lipids, lipoproteins and apolipoproteins (inset) (n=9-12, each group) and FPLC analysis of pooled plasma (n=5,
Contribution of hepatic ABCG5/G8 overexpression to sterol transport and atherosclerosis

each group) from female LDLr-KO and liver G5/G8-Tg x LDLr-KO mice on RCD and Western diet. Panel D. Plasma cholesterol, campesterol and sitosterol concentrations and plant sterol/cholesterol ratios in 3-month-old female LDLr-KO (white bars) and liver G5/G8-Tg x LDLr-KO (black bar) mice (n=4-6, each group) on RCD. Panel E. Mean proximal aortic lesion area in male and female LDLr-KO and liver G5/G8-Tg x LDLr-KO mice placed on a Western diet for 10 and 13 weeks.

Fig. 5. Effect of Liver ABCG5/G8 Overexpression on the Hepatic Cholesterol Balance in ApoE-KO (Left Panel) and LDLr-KO (Right Panel) Mice. The amount of cholesterol ingested daily (Dietary Cholesterol) is similar in liver G5/G8-Tg x E-KO vs E-KO mice and liver G5/G8-Tg x LDLr-KO vs LDLr-KO mice. Biliary cholesterol secretion (Bile Cholesterol) is increased in Tg x E-KO vs E-KO mice (by 1.4-fold) and in Tg x LDLr-KO vs LDLr-KO mice (by 1.7-fold). Thus, the amount of intestinal cholesterol available for absorption (Intestinal Cholesterol=Dietary Cholesterol + Bile Cholesterol) is greater in Tg x E-KO vs E-KO mice (by 1.3-fold) and Tg x LDLr-KO vs LDLr-KO (1.3-fold). Hepatic ABCG5/G8 overexpression did not alter fecal cholesterol excretion in E-KO or LDLr-KO mice; hence, the daily amount of absorbed cholesterol was increased in liver G5/G8-Tg x E-KO vs E-KO mice (1.5-fold) and in liver G5/G8-Tg x LDLr-KO vs LDLr-KO mice (1.4-fold). Thus, in liver G5/G8-Tg mice the hepatic output of cholesterol (via bile) is increased over controls; however, the input of cholesterol into the liver (from intestinal cholesterol absorption and transport via chylomicrons) is also increased. Consequently, the cholesterol balance across the liver of G5/G8-Tg x E-KO vs E-KO mice and G5/G8-Tg x LDLr-KO vs LDLr-KO mice was not significantly altered by hepatic overexpression of ABCG5/G8. Liver cholesterol concentrations, hepatic expression of SR-BI, LDLr, LRP, Cyp7, HMGCoA reductase and plasma lipoprotein levels were unchanged in liver G5/G8-Tg x E-KO vs E-KO and liver G5/G8-Tg x LDLr-KO vs LDLr-KO mice. Dietary cholesterol, biliary cholesterol secretion and fecal cholesterol excretion were directly measured. Intestinal cholesterol (=diet cholesterol + bile cholesterol) and absorbed cholesterol (=intestinal cholesterol – fecal cholesterol excretion) were determined from these measured values. Data is expressed as mg of cholesterol/day/30 gm BW for E-KO and LDLr-KO (C) vs liver G5/G8-Tg x E-KO and
Contribution of hepatic ABCG5/G8 overexpression to sterol transport and atherosclerosis

liver G5/G8-Tg x LDLr-KO (Tg).

Reference List


Contribution of hepatic ABCG5/G8 overexpression to sterol transport and atherosclerosis


Figure 1
Figure 2

A

LIVER

<table>
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<th>RCD</th>
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SMALL INTESTINE

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<td>G8</td>
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<tr>
<td>C</td>
<td>Tg</td>
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β-actin

B

C

mg/dL

TC  | TG  | PL  | CE  | FC  | HDL-C

mg/dL

Cholest.

Camp.

Sitost.

 Plasma Sterol Ratio x 10^3

Figure 2
Figure 3
Figure 4
### TABLE I: Hepatobiliary Sterol Concentrations, Sterol Secretion Rates and Intestinal Cholesterol Absorption in Liver G5/G8-Tg and Control Mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Diet</th>
<th>n</th>
<th>Bile Volume</th>
<th>Biliary Sterol Concentration</th>
<th>Biliary Sterol Secretion Rate</th>
<th>Dietary Cholesterol</th>
<th>Intestinal Cholesterol (Diet + Bile) Absorption %</th>
<th>Absorbed Cholesterol</th>
<th>Fecal Cholesterol Excretion</th>
<th>Hepatic Sterols</th>
<th>n</th>
<th>uM/hr/100gm</th>
<th>um/hr/kg</th>
<th>mg/d/30gm</th>
<th>mg/d/30gm</th>
<th>mg/d/30gm</th>
<th>mg/d/30gm</th>
<th>ug/mg liver</th>
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<td>A</td>
<td>C57Bl/6 RCD</td>
<td>28</td>
<td>305±11</td>
<td>772.20±39.27</td>
<td>2.36±0.15</td>
<td>0.73±0.04</td>
<td>1.10±0.20</td>
<td>1.83±0.20</td>
<td>40.20±2.00</td>
<td>0.74±0.21</td>
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<td></td>
<td>Tg</td>
<td>16</td>
<td>291±12</td>
<td>830.17±76.90</td>
<td>2.35±0.17</td>
<td>0.73±0.05</td>
<td>1.10±0.30</td>
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<td>0.71±0.21</td>
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<tr>
<td>B</td>
<td>C57Bl/6 4%C</td>
<td>13</td>
<td>355±20</td>
<td>993.70±17.30</td>
<td>3.63±0.07</td>
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<td>171.86±3.37</td>
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<td>6.73±0.31**</td>
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<td>170.73±3.38</td>
<td>172.81±3.38</td>
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<tr>
<td>C</td>
<td>ApoE-KO RCD</td>
<td>16</td>
<td>357±18</td>
<td>1689.47±38.09</td>
<td>6.12±0.20</td>
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<td>Tg x E-KO</td>
<td>8</td>
<td>312±25</td>
<td>2602.87±63.58**</td>
<td>8.84±0.41**</td>
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<td>1.20±0.06</td>
<td>3.94±0.14*</td>
<td>75.87±1.23</td>
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<td>D</td>
<td>LDLr-KO Western</td>
<td>9</td>
<td>325±30</td>
<td>2598.61±154.12</td>
<td>7.94±0.94</td>
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<td>367±29</td>
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Biliary sterol concentrations and secretion rates were significantly increased in liver G5/G8-Tg on the 4%C diet, in liver G5/G8-Tg x E-KO mice on a RCD and in liver G5/G8-Tg x LDLr-KO on a Western diet. Consequently, the amount of intestinal cholesterol available for absorption (Intestinal Cholesterol = Dietary Cholesterol + Bile Cholesterol) and the amount of cholesterol absorbed daily (Absorbed Cholesterol) was greater in liver G5/G8-Tg x E-KO mice and in liver G5/G8-Tg x LDLr-KO vs LDLr-KO mice. Since hepatic cholesterol output (via bile) and input (from intestinal absorption) were both increased in Tg x E-KO and Tg x LDLr-KO mice, the balance of cholesterol across the liver was not significantly altered in these mice. Hepatic cholesterol concentrations were similar between all liver G5/G8-Tg and control group mice. There was no significant difference in the volume of bile collected per hour (Bile Volume), in the amount of dietary cholesterol ingested daily (Dietary Cholesterol), in the fractional intestinal cholesterol absorption nor in the daily fecal cholesterol excretion between any of the liver G5/G8-Tg and control mouse groups. The number of mice cannulated for bile sterol analysis in each study group is indication (n). The number of mice utilized for measurement of fractional cholesterol absorption (n=6-17) and fecal cholesterol excretion (n=5-6) in each study group is indicated in Results. Data is expressed as mean +/- SEM per 30 gm or 1 Kg BW. BW. +p<0.04, ++p<0.002, *p<0.006, **p<0.0005.
Hepatic ABCG5 and ABCG8 overexpression increases hepatobiliary sterol transport but alter aortic atherosclerosis in transgenic mice

Justina E Wu, Federica Basso, Robert D Shamburek, Marcelo J A Amar, Boris Vaisman, Gergeley Szakacs, Charles Joyce, Terese Tansey, Lita Freeman, Beverly J Paigen, Fairwell Thomas, H. Bryan Brewer, Jr. and Silvia Santamarina-Fojo

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