Increased fecal neutral sterol loss upon liver-X-receptor activation is independent of biliary sterol secretion in mice

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* Equally contributed to this study

Submitted.
Chapter 5

Summary

Reverse cholesterol transport (RCT) is defined as high-density lipoprotein (HDL)-mediated flux of excess cholesterol from peripheral cells to liver, followed by secretion into bile and disposal via the feces. Various steps of this pathway are controlled by the liver X receptor (LXR). We addressed the role of the intestine in LXR-dependent stimulation of fecal cholesterol excretion. To segregate biliary from intestine-derived cholesterol, wild-type and Mdr2 P-glycoprotein deficient mice (Mdr2\(^{-/-}\)), which are unable to secrete cholesterol into bile, were treated with the LXR agonist GW3965. Treatment with GW3965 increased biliary cholesterol secretion by 74\% in wild-type mice, but had no effect in Mdr2\(^{-/-}\) mice. LXR activation increased fecal neutral sterol excretion 2.1-fold in wild-type mice. Surprisingly, an identical increase was observed in Mdr2\(^{-/-}\) mice. Fractional cholesterol absorption was reduced upon LXR activation in both strains, but more pronounced in Mdr2\(^{-/-}\) mice coinciding with reduced Npc1l1 expression. Intestinal gene expression of Abca1, Abcg1, Abcg5 and Abcg8 was strongly induced upon LXR activation in both strains, while expression of HMGC\(\text{CoA}\) reductase, controlling cholesterol synthesis, remained unaffected. Additionally, LXR activation stimulated the excretion of plasma-derived [\(^{3}\text{H}\)]cholesterol into the fecal neutral sterol fraction in Mdr2\(^{-/-}\) mice. Increased fecal cholesterol loss upon LXR activation is independent of biliary cholesterol secretion in mice. An important part of excess cholesterol is excreted directly via the intestine, supporting the existence of an alternative, quantitatively important route for cholesterol disposal.
Fecal sterol loss independent of bile secretion

Introduction

Cholesterol accumulation in macrophages (foam cells) in the arterial vessel wall is considered a primary event in the development of atherosclerosis. Removal of excess cholesterol from these cells, as well as from other peripheral cells, is therefore of crucial importance. This pathway, referred to as reverse cholesterol transport (RCT), is usually defined as the HDL-mediated flux of cholesterol from peripheral cells to the liver, followed by its secretion into bile and disposal via the feces. The ATP-binding cassette (ABC) transporter A1 (ABCA1) facilitates the obligatory first step of RCT, i.e., the efflux of cholesterol from peripheral cells towards HDL. HDL-cholesterol is subsequently taken up by the liver, mainly via scavenger receptor-BI (SR-BI), and finally may be excreted into bile, either as free cholesterol or after conversion to bile salts. Hepatobiliary elimination of cholesterol was shown to be mediated, at least partially, by the half-transporters ABCG5 and ABCG8. Part of biliary cholesterol which mixes with dietary cholesterol in the lumen of the small intestine is taken up by the Niemann-Pick C1 Like 1 (NPC1L1) protein, which has recently been shown to play a role in cholesterol absorption, and transported back to the liver by the chylomicron-remnant pathway. Another part, however, is lost into feces. It has become clear that cholesterol absorption is not a passive process, but depends on the combined actions of transporter proteins involved in uptake (NPC1L1) and efflux (ABCG5, ABCG8).

Fecal excretion of cholesterol in mice can be enhanced via activation of the nuclear liver X receptor (LXR), for which oxysterols have been identified as natural ligands. LXR regulates expression of a number of genes crucially involved in RCT, including the members of the ATP-binding cassette transporter family mentioned above. Activation of LXR in mice leads to elevated HDL levels, increased biliary cholesterol excretion, reduced intestinal cholesterol absorption efficiency and, finally, to increased neutral sterol loss via the feces.

Recent data, however, indicate that the concept of RCT requires extensive re-thinking. Overexpression of cholesteryl ester-transfer protein (CETP) in mice, resulting in low HDL levels, showed that neither the HDL levels nor the level of CETP activity dictated the magnitude of centripetal cholesterol flux (i.e., RCT) to the liver in mice. Studies in ApoA-1−/− mice, which have strongly decreased plasma HDL, revealed that neither HDL nor ApoA-1 levels are important determinants of centripetal cholesterol flux. More recently, we reported that the absence of plasma HDL, and thus of HDL-mediated RCT, in Abca1-null mice does not at all affect hepatobiliary cholesterol transport.

Recent data from our laboratory indicate that, besides the liver, the intestine may play a role as excretory organ in RCT: upon pharmacological LXR activation, fecal cholesterol excretion in various mouse models (C57BL/6J, DBA/1, Abca1−/− on a DBA/1 background) was increased to a much larger extent than could be explained by stimulation of biliary cholesterol secretion alone. To resolve the apparent discrepancies between the "classical" biliary route for removal of excess cholesterol and these findings, we now used an animal
model in which biliary cholesterol secretion is strongly reduced, \textit{i.e.}, Mdr2 P-glycoprotein (Pgp)-deficient (\textit{Mdr2}\textsuperscript{--/--}) mice. Mdr2 (or \textit{Abcb4} according to new nomenclature) mediates the ATP-dependent translocation of phospholipids at the canalicular membrane of hepatocytes. Consequently, Mdr2 P-glycoprotein deficiency in mice leads to an inability to secrete phospholipids into the bile. Due to the tight coupling of phospholipid and cholesterol excretion, these mice also show a strongly impaired biliary cholesterol secretion.\textsuperscript{7,8}

The first question we addressed was whether LXR activation, known to strongly increase biliary cholesterol output in wild-type mice,\textsuperscript{3} would stimulate biliary cholesterol secretion in a phospholipid-independent manner. For this purpose, \textit{Mdr2}\textsuperscript{+/-} and wild-type mice were treated with the synthetic LXR agonist GW3965.\textsuperscript{9} Upon GW3965 treatment, biliary cholesterol secretion remained nearly undetectable in \textit{Mdr2}\textsuperscript{--/--} mice, whereas it increased in wild-type mice. This allowed us to assess whether activation of LXR would stimulate fecal neutral sterol output independent of its effects on biliary cholesterol secretion. It was found that wild-type and \textit{Mdr2}\textsuperscript{+/-} mice treated with a synthetic LXR agonist showed a similarly increased fecal neutral sterol output without induction of genes involved in cholesterol synthesis, indicating that this increase must result from intestinal sources. This conclusion was supported by the finding that \textit{Mdr2}\textsuperscript{--/--} mice showed increased secretion of plasma-derived radio-labeled cholesterol into the feces upon treatment with the LXR agonist. We therefore postulate that, besides the liver, the intestine plays an important and up to now underestimated role in cholesterol disposal.

\section*{Materials and Methods}

\subsection*{Animals and diet}
Female \textit{Mdr2}\textsuperscript{+/-} mice (4-6 months) on an FVB background were obtained from the Central Animal Facility, Academic Medical Center, Amsterdam, The Netherlands. Age-matched FVB wild-type mice were purchased from Harlan (Horst, The Netherlands). Mice received standard mouse chow containing 0.017 w/w \% cholesterol (Arie Blok, Woerden, The Netherlands), or chow supplemented with the synthetic LXR agonist GW3965, 35 mg/kg/day (based on 26 g body weight and consumption of 3 g chow/day) for 10 days. GW3965 was kindly provided by GlaxoSmithKline, Stevenage, UK. All experiments were performed with the approval of the Ethical Committee for Animal Experiments of the University of Groningen.
Experimental methods

Animals were housed in groups and feces were collected upon day 8 until day 10 of treatment. After 10 days, animals were anaesthetized by intraperitoneal injection of Hypnorm (fentanyl/fluanisone, 1 ml/kg) and diazepam (10 mg/kg). After puncturing the gallbladder and disposal of its content, hepatic bile was collected for 30 minutes from the common bile duct via the gallbladder. During bile collection, body temperature was stabilized using a humidified incubator. Bile flow was determined gravimetrically assuming a density of 1 g/ml for bile. After bile collection, animals were killed by cardiac puncture. Blood was collected in EDTA-containing tubes. Livers were excised and weighed. The small intestine was rinsed with cold phosphate-buffered saline containing 100 µM phenylmethylsulforyl fluoride (PMSF) and divided into three equal parts. Parts of both the liver and intestine were snap-frozen in liquid nitrogen and stored at -80°C for mRNA isolation and biochemical analysis. Samples for microscopic evaluation were frozen in isopentane and stored at -80°C, or fixed in paraformaldehyde, for hematoxylin/eosin, oil red O and Ki-67 staining.

Fractional cholesterol absorption was measured in a separate experiment using the fecal dual-isotope method. Animals were housed individually one week prior to the experiment. Wild-type and *Mdr2*<sup>-/-</sup> mice were fed normal chow or chow supplemented with GW3965 (35 mg/kg/day). After 6 days, mice received by gavage 150 µl of medium-chain triglyceride oil containing 1µCi of [<sup>14</sup>C]cholesterol (Amersham Bioscience, Buckinghamshire, UK) and 2 µCi of [<sup>3</sup>H]sitostanol (American Radiolabeled Chemicals, St Louis, USA). Feces were collected for the next 4 days. After 4 days animals were sacrificed and the small intestine was removed. The intestinal contents were flushed with saline containing 0.5 mM taurocholate and radioactivity was measured upon dissolving the intestine in NCS-TS (Amersham Bioscience).

For determination of cholesterol kinetics, only *Mdr2*<sup>-/-</sup> mice were used. Mice were housed individually one week prior to the experiment. Mice were fed normal chow or chow supplemented with GW3965 (35 mg/kg/day). After 8 days, mice received an intravenous injection of 1.7 µCi [<sup>3</sup>H]cholesterol (NEN Life Science, Zaventem, Belgium) dissolved in Intralipid (20%, Fresenius Kabi, Den Bosch, The Netherlands) via retro-orbital injection. Blood samples (75µl) were drawn at day 1, 2, 3 and 4 after injection by orbita puncture. Feces were collected from individual mice at day 1, 2, 3 and 4 after injection. After 4 days, bile was collected as described above and animals were sacrificed afterwards. Liver and intestine were collected for radioactivity measurement.

Analytical methods

Bile salts were measured enzymatically. Commercially available kits were used for the determination of free cholesterol (Wako, Neuss, Germany), total cholesterol and triglycerides (Roche Molecular Biochemicals, Mannheim, Germany) and phospholipids (Wako) in plasma. From each group, plasma samples were pooled and used for lipoprotein separation by fast protein liquid chromatography (FPLC) on a Superose 6B 10/30 column (Amersham Bio-
science). Livers were homogenized and hepatic lipids were extracted according to Bligh and Dyer.\textsuperscript{11} Hepatic triglyceride and cholesterol concentrations were determined using commercially available kits (Roche, Wako). Phospholipids in bile and liver were determined as described by Böttcher \textit{et al.}\textsuperscript{12} Cholesterol in bile was measured according to Gamble \textit{et al.}\textsuperscript{13} Feces were weighed and homogenized. Neutral sterols and bile salts were analyzed according to Acra \textit{et al.}\textsuperscript{14} and Setchell \textit{et al.}\textsuperscript{15}

Feces collected for cholesterol absorption measurements after oral administration of [\textsuperscript{3}H]sitostanol and [\textsuperscript{14}C]cholesterol were freeze-dried, saponified, and neutral sterols were extracted. The [\textsuperscript{14}C] and [\textsuperscript{3}H] content of the feces and dosing mixture were counted and the ratio was used to calculate the percentage cholesterol absorption:

\[
\text{% cholesterol absorption} = \left( \frac{\text{[\textsuperscript{14}C] / [\textsuperscript{3}H] dosing mixture} - \text{[\textsuperscript{14}C] / [\textsuperscript{3}H] feces}}{\text{[\textsuperscript{14}C] / [\textsuperscript{3}H] dosing mixture}} \right) \times 100
\]

To measure kinetics of fecal disposal of plasma-derived [\textsuperscript{3}H]cholesterol, the [\textsuperscript{3}H] content of plasma (10 µl) was measured by liquid scintillation counting. Before counting the [\textsuperscript{3}H] content of feces, feces were freeze-dried, saponified, and neutral sterols were extracted according to Bligh and Dyer.\textsuperscript{11}

\textbf{Histology}

Liver and small intestine were examined after hematoxilin-eosin (HE) staining and oil red-o (ORO) staining for neutral lipids. HE staining was performed on paraformaldehyde-fixed, paraffin-embedded sections by standard procedures. Oil-red-o staining was performed on frozen sections by standard procedures.

\textbf{Immunohistochemical staining of intestine}

Ki-67 staining was performed on paraformaldehyde-fixed, paraffin-embedded intestinal sections. Endogenous peroxidase activity was blocked by incubation of sections in 0.3% (v/v) H\textsubscript{2}O\textsubscript{2} in 30% (v/v) methanol for 4 minutes. Sections were pretreated with 10 nM citrate buffer (pH 6) for 8 minutes at 100 °C. Nonspecific binding was blocked by pre-incubation with normal goat serum. Sections were incubated with Ki-67 polyclonal antibody (Novo Castra, Newcastle, UK) (1:500) for 60 minutes. Secondary antibody against rabbit (IgG) labeled with biotine (Dako, Glostrup, Denmark) (1:100, 30 min.) was used. Antibody binding was visualized using streptavidine labeled with peroxidase (Dako), DAB (Sigma, St. Louis, USA) and peroxide. Sections were counterstained with hematoxylin.

\textbf{Immunochemical staining of Abca1 and Abcg5 in the small intestine}

For homogenates and brush border membrane isolation, mid sections of the small intestines were used. Mucosa was scraped before homogenization in buffer containing 250 mM sucrose,
10 mM Tris-base (pH 7.4), and a mixture of protease inhibitors containing 100 µM PMSF and Complete (Roche Diagnostics, Almere, The Netherlands). From these homogenates, brush border membrane (BBM) fractions were isolated by calcium precipitation as described by Schmitz et al.\textsuperscript{16} Enrichment of BBM fractions was determined by alkaline phosphatase activity measurements according to Keeffe et al.\textsuperscript{17} Protein was determined according to Lowry et al.\textsuperscript{18} Homogenates (50 µg protein) for detection of Abca1 and BBM fractions (15 µg protein, corrected for enrichment) for detection of Abcg5 were electrophoresed through polyacrylamide gels and blotted on nitrocellulose membranes. Membranes were blocked in Tris-buffered saline (pH 7.4) containing 1% Tween 20 and 5% skim milk powder. Membranes were incubated with anti-Abca1 antibody,\textsuperscript{19} kindly provided by M. Hayden, (1:1000 in blocking buffer), or Abcg5 antiserum\textsuperscript{20} (1:1500 in blocking buffer) for 2 hours at room temperature. After washing, immunocomplexes were detected using horseradish peroxidase-conjugated sheep anti-mouse IgG (Abca1) or goat ant-rabbit IgG (Abcg5) and ECL detection (All three from Amersham Bioscience, Little Chalfont, UK).

RNA isolation and PCR procedures
Total RNA was isolated with Trizol (Invitrogen, Parsley, UK) and quantified using Ribogreen (Molecular Probes, Eugene, USA). Equal amounts of RNA from the three distinct parts of the small intestine were pooled prior to reverse transcription. Real-time quantitative PCR was performed using an Applied Biosystems 7700 Sequence detector according to the manufacturer’s instructions. Primers were obtained from Invitrogen. 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 (Abca1, Abcg5, Abcg8, Acat2, Hmgcr and Sr-b1,\textsuperscript{3} except Abcg1 (NM_009593; forward CAAGACCCCTTTTGAAGGGATCTC, reverse GCCAGAATTATTGATGTCCGAC, probe CCCATGAATGCCCACAGCTCTCC), Abcg2 (AF140218; forward AATCAGGGCATCGAACTGTCA, reverse CAGGTAGGCAATTGTGAGGAGA, probe CAAGCCAGGGCCACATGATTCTTC) and Npc1l1 (AY437866; forward GAGAGCCAAAGATGCTACTATTTCA, reverse CCCGGAAGTTGTCATG, probe ACTCCAGCAAACACCGCACTGCC). All expression data were subsequently standardized for β-actin RNA, which was analyzed in separate runs.

Statistics
Statistical analyses were performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, USA). Differences between the groups were analyzed by Kruskal-Wallis test followed by Mann-Whitney-U-test. A p-value smaller than 0.05 was considered statistically significant.
Results

Plasma and liver parameters upon LXR activation in wild-type and Mdr2\(^{-/-}\) mice

Treatment with the LXR agonist GW3965 led to a significant increase in plasma cholesterol levels in wild-type mice (Table I). Plasma cholesterol levels also increased upon treatment in the Mdr2\(^{-/-}\) mice, with basal plasma cholesterol levels of 36% of wild-type values (Table I). The increase in plasma cholesterol in both strains was mainly confined to the HDL-sized fractions, as shown by FPLC analysis (Figure I). Plasma triglyceride concentrations were not significantly affected by GW3965 treatment in wild-type or Mdr2\(^{-/-}\) mice (Table I).

Although it has been reported that treatment of C57BL/6J mice with the LXR agonist T0901317 has a profound effect on liver weight,\(^{21}\) no significant change in liver weight was seen in FVB mice after treatment with GW3965 (Table I). Mdr2\(^{-/-}\) mice had an increased liver weight compared to wild-type mice (+ 71%), in agreement with earlier reports.\(^{22}\) Bile duct proliferation was observed in all Mdr2\(^{-/-}\) mice, but in none of the wild-type mice (data not shown). As shown earlier with the LXR agonist T0901317 by us\(^{21}\) and others,\(^{23}\) GW3965 treatment also led to an increased triglyceride content in the liver of wild-type mice (+ 106%). Surprisingly, no increase was seen in Mdr2\(^{-/-}\) mice upon GW3965 treatment, as confirmed by oil red-o staining (data not shown). GW3965 treatment did not lead to changes in liver cholesterol levels in wild-type or Mdr2\(^{-/-}\) mice.

Table I: Plasma and liver lipid levels of wild-type and Mdr2\(^{-/-}\) mice maintained on chow diet in the presence or absence of the LXR agonist GW3965.

<table>
<thead>
<tr>
<th></th>
<th>wild-type control</th>
<th>wild-type GW3965</th>
<th>Mdr2(^{-/-}) control</th>
<th>Mdr2(^{-/-}) GW3965</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma cholesterol (mM)</td>
<td>2.34 ± 0.41</td>
<td>3.22 ± 0.33 (a)</td>
<td>0.85 ± 0.20 (a)</td>
<td>1.36 ± 0.11 (b)</td>
</tr>
<tr>
<td>Plasma triglycerides (mM)</td>
<td>2.31 ± 0.67</td>
<td>2.76 ± 0.39</td>
<td>1.67 ± 0.28</td>
<td>1.77 ± 0.34</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>25.8 ± 3.3</td>
<td>25.7 ± 3.6</td>
<td>26.3 ± 1.6</td>
<td>26.9 ± 2.8</td>
</tr>
<tr>
<td>Liver weight (% of body weight)</td>
<td>4.51 ± 0.40</td>
<td>4.79 ± 0.23</td>
<td>7.72 ± 0.75 (a)</td>
<td>9.47 ± 1.02 (b)</td>
</tr>
<tr>
<td>Liver cholesterol (nm/mg liver)</td>
<td>6.00 ± 1.19</td>
<td>5.33 ± 1.19</td>
<td>6.67 ± 0.98</td>
<td>5.79 ± 0.53</td>
</tr>
<tr>
<td>Liver phospholipids (nmol/mg liver)</td>
<td>31.37 ± 3.16</td>
<td>34.38 ± 4.96</td>
<td>29.92 ± 4.08</td>
<td>25.96 ± 1.04 (b)</td>
</tr>
<tr>
<td>Liver triglycerides (nmol/mg liver)</td>
<td>12.83 ± 4.87</td>
<td>26.43 ± 5.91 (a)</td>
<td>4.55 ± 1.23 (a)</td>
<td>4.58 ± 1.08</td>
</tr>
</tbody>
</table>

Female FVB wild-type (n = 7 per group) and Mdr2\(^{-/-}\) mice (n = 6 per group) were fed a chow diet in the presence or absence of GW3965 (35 mg/kg/d) for 10 days. Blood was collected by cardiac puncture. Livers were collected and frozen immediately. Analysis of lipids was performed as described in MATERIALS AND METHODS. Values represent means ± S.D. \(^a\)Indicates significant difference (Kruskal-Wallis test followed by Mann-Whitney U test, \(p<0.01\)) compared to control wild-type mice. \(^b\) Indicates significant difference (Kruskal-Wallis test followed by Mann-Whitney U test, \(p<0.01\)) compared to control Mdr2\(^{-/-}\) mice.
Fecal sterol loss independent of bile secretion

Figure 1: Effect of LXR activation on distribution of cholesterol in plasma lipoprotein fractions of wild-type and Mdr2\(^{-/-}\) mice.

FPLC analysis of plasma cholesterol of female FVB wild-type (A) and Mdr2\(^{-/-}\) (B) mice. Mice were maintained for 10 days on chow diet in the presence (closed circles) or absence (open circles) of the LXR agonist GW3965 (35 mg/kg/d). Blood was collected via cardiac puncture and pooled before FPLC analysis. Analysis was performed as described in MATERIALS AND METHODS.

Biliary secretion rates upon LXR activation

We have previously shown that LXR stimulation enhances cholesterol secretion without increasing phospholipid secretion into bile, resulting in uncoupling of biliary cholesterol and phospholipid secretion.\(^3\) Treatment with GW3965 increased biliary cholesterol secretion 1.7-fold in wild-type mice, whereas bile flow, biliary bile salt and phospholipid secretion were not affected (Table II). As a result, the ratio of cholesterol to phospholipids doubled upon GW3965 treatment. Mdr2\(^{-/-}\) mice, compared to wild-type mice, showed an increased bile flow, similar to earlier reports\(^2\) and a severely decreased biliary cholesterol secretion. Upon GW3965 treatment biliary cholesterol secretion remained unaffected. As expected, phospholipids in bile of Mdr2\(^{-/-}\) mice were not detectable (Table II).
Table II: Biliary output parameters of wild-type and Mdr2⁻/⁻ mice maintained on a chow diet in the presence or absence of the LXR agonist GW3965.

<table>
<thead>
<tr>
<th></th>
<th>wild-type control</th>
<th>wild-type GW3965</th>
<th>Mdr2⁻/⁻ control</th>
<th>Mdr2⁻/⁻ GW3965</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow (µl/min/100 g body weight)</td>
<td>4.7 ± 1.0</td>
<td>4.8 ± 1.0</td>
<td>7.4 ± 1.3</td>
<td>10.0 ± 3.3</td>
</tr>
<tr>
<td>Bile salts (nmol/min/100 g bw)</td>
<td>293 ± 97</td>
<td>318 ± 137</td>
<td>395 ± 67</td>
<td>549 ± 351</td>
</tr>
<tr>
<td>Cholesterol (nmol/min/100 g bw)</td>
<td>1.9 ± 0.6</td>
<td>3.3 ± 1.1</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Phospholipid (nmol/min/100 g bw)</td>
<td>52.1 ± 11.6</td>
<td>44.2 ± 15.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cholesterol/phospholipid ratio</td>
<td>0.04 ± 0.02</td>
<td>0.08 ± 0.02</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Female FVB wild-type (n = 7 per group) and Mdr2⁻/⁻ mice (n = 6 per group) were fed a chow diet in the presence or absence of GW3965 (35 mg/kg/d) for 10 days. Bile was collected for 30 min. Analysis of bile was performed as described in MATERIALS AND METHODS. Phospholipids in bile in Mdr2⁻/⁻ mice were not detectable (ND). Values represent means ± S.D. * Indicates significant difference (Kruskal-Wallis test followed by Mann-Whitney U test, p<0.01) compared to control wild-type mice. † Indicates significant difference (Kruskal-Wallis test followed by Mann-Whitney U test, p<0.01) compared to control Mdr2⁻/⁻ mice.

Fecal sterol balance

Neutral sterols and bile salts were determined in the feces (Figure 2). Fecal neutral sterol output was increased by 2.1-fold in wild-type mice after GW3965 treatment. Surprisingly, Mdr2⁻/⁻ mice showed a similar 2.1-fold increase in fecal neutral sterol loss as wild-type mice after GW3965 treatment. There was no change in fecal bile salt output upon LXR activation in either strain.

Figure 2: Fecal loss of bile salts and neutral sterols of wild-type and Mdr2⁻/⁻ mice maintained on chow diet in the absence or presence of the LXR agonist GW3965.

Female FVB wild-type and Mdr2⁻/⁻ mice were maintained on chow diet in the absence (white bars) or presence (black bars) of the LXR agonist GW3965 (35 mg/kg/d) for 10 days. Animals were housed in groups during the experiment and feces were collected last 48 hour of experiment. Feces were analysed as described in MATERIALS AND METHODS. * Indicates significant difference (Mann-Whitney U test, p<0.05).
**Cholesterol absorption**

GW3965 treatment reduced fractional cholesterol absorption as measured by the oral dual isotope test in wild-type mice by about 50% (Figure 3). Surprisingly, fractional cholesterol absorption in untreated $Mdr2^{-/-}$ mice was similar to that in wild-type mice. Upon LXR activation, however, fractional cholesterol absorption in $Mdr2^{-/-}$ decreased drastically to undetectable levels. The recovery of $[^{14}C]$cholesterol in the small intestinal wall at 4 days after its intragastric administration was similar in untreated wild-type and $Mdr2^{-/-}$ mice (3.9 ± 1.2% of dose in wild-type and 3.3 ± 0.2% in $Mdr2^{-/-}$ mice). LXR activation did not reduce this percentage in wild-type mice (3.1 ± 0.7% of dose), however, $Mdr2^{-/-}$ mice showed a strongly reduced recovery of $[^{14}C]$cholesterol in the intestinal wall (0.8 ± 0.4% of dose).

**Figure 3:** Fractional cholesterol absorption of wild-type and $Mdr2^{-/-}$ mice maintained on chow diet in the absence or presence of the LXR agonist GW3965. Female FVB wild-type and $Mdr2^{-/-}$ mice were maintained on chow diet in the absence (white bars) or presence (black bars) of the LXR agonist GW3965 (35 mg/kg/d) for 10 days. Animals were housed individually. Fractional cholesterol absorption was measured using the fecal dual-isotope method as described in MATERIALS AND METHODS. * Indicates significant difference (Mann-Whitney U test, p<0.05).

**mRNA and protein expression in the small intestine**

As the results described above suggest involvement of the intestine in cholesterol excretion into the feces, we determined expression levels of several genes involved in cholesterol metabolism in the small intestine of wild-type and $Mdr2^{-/-}$ mice upon LXR activation. LXR activation by GW3965 treatment resulted in a more than 2-fold induction of $Abcg5$ and $Abcg8$ mRNA levels in the small intestine in both strains (Table III), in agreement with increased protein expression of Abcg5 (Figure 4A). In addition, LXR activation led to a more than 9-fold increase in $Abca1$ mRNA levels (Table III) and concomitant increase of Abca1 protein (Figure 4B) in the small intestine in both strains. $Abcg1$ mRNA expression in the small intestine was more than 12-fold induced after LXR stimulation in both strains (Table III).
Expression of \( Sr-b1 \) increased over 2-fold in both strains after LXR activation (Table III). In contrast, intestinal expression of \( Npc1l1 \), recently identified as intestinal cholesterol absorption protein,\(^2\) tended to decrease upon LXR activation in wild-type mice and was significantly decreased under these conditions in \( Mdr2^{-/-} \) mice. Expression of \( Hmgcr \) and \( Acat2 \), indicative of intestinal cholesterol synthesis and esterification, remained unaffected upon GW3965 treatment in wild-type and \( Mdr2^{-/-} \) mice (Table III).

To test whether increased intestinal cell proliferation might contribute to enhanced fecal cholesterol loss, Ki-67 staining was performed to visualize dividing cells. Visual inspection of Ki-67 stained intestinal sections, however, did not reveal indications for increased cell proliferation upon LXR activation (data not shown).

### Table III: mRNA expression levels in small intestine of wild-type and \( Mdr2^{-/-} \) mice treated with or without the LXR agonist GW3965.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>wild-type control</th>
<th>wild-type GW3965</th>
<th>Mdr2^{-/-} control</th>
<th>Mdr2^{-/-} GW3965</th>
</tr>
</thead>
<tbody>
<tr>
<td>( Abca1 )</td>
<td>1.00 ± 0.41</td>
<td>9.51 ± 2.81 ( ^a )</td>
<td>0.44 ± 0.15 ( ^a )</td>
<td>4.40 ± 0.96 ( ^\beta )</td>
</tr>
<tr>
<td>( Abcg1 )</td>
<td>1.00 ± 0.22</td>
<td>13.31 ± 4.48 ( ^a )</td>
<td>0.83 ± 0.24</td>
<td>10.03 ± 3.96 ( ^\beta )</td>
</tr>
<tr>
<td>( Abcg2 )</td>
<td>1.00 ± 0.30</td>
<td>1.42 ± 0.57</td>
<td>0.93 ± 0.40</td>
<td>1.21 ± 0.23</td>
</tr>
<tr>
<td>( Abcg5 )</td>
<td>1.00 ± 0.24</td>
<td>3.12 ± 1.21 ( ^a )</td>
<td>0.69 ± 0.17 ( ^a )</td>
<td>1.94 ± 0.74 ( ^\beta )</td>
</tr>
<tr>
<td>( Abcg8 )</td>
<td>1.00 ± 0.30</td>
<td>2.88 ± 0.81 ( ^a )</td>
<td>0.82 ± 0.36</td>
<td>1.79 ± 0.58 ( ^\beta )</td>
</tr>
<tr>
<td>( Sr-b1 )</td>
<td>1.00 ± 0.38</td>
<td>2.19 ± 0.80 ( ^a )</td>
<td>0.55 ± 0.18 ( ^a )</td>
<td>1.71 ± 0.71 ( ^\beta )</td>
</tr>
<tr>
<td>( Npc1l1 )</td>
<td>1.00 ± 0.42</td>
<td>0.71 ± 0.20</td>
<td>1.06 ± 0.21</td>
<td>0.47 ± 0.14</td>
</tr>
<tr>
<td>( Hmgcr )</td>
<td>1.00 ± 0.19</td>
<td>1.19 ± 0.45</td>
<td>0.92 ± 0.43</td>
<td>1.04 ± 0.41</td>
</tr>
<tr>
<td>( Acat2 )</td>
<td>1.00 ± 0.30</td>
<td>1.07 ± 0.26</td>
<td>0.89 ± 0.27</td>
<td>1.07 ± 0.44</td>
</tr>
</tbody>
</table>

Female wild-type FVB and \( Mdr2^{-/-} \) mice were maintained on chow diet in the absence or presence of the LXR agonist GW3965 (35 mg/kg/d) for 10 days. RNA was isolated from 3 parts of the small intestine; equal amounts of RNA were pooled before synthesis of cDNA. Synthesis of cDNA and quantitative real-time PCR was performed as described in MATERIALS AND METHODS. All data were standardised for \( \beta\)-actin RNA. Expression in wild-type mice on control diet was set to 1.00. Values represent means ± S.D. \( ^a \) Indicates significant difference (Kruskal-Wallis test followed by Mann-Whitney U test, \( p<0.01 \)) compared to control wild-type mice. \( ^\beta \) Indicates significant difference (Kruskal-Wallis test followed by Mann-Whitney U test, \( p<0.01 \)) compared to control \( Mdr2^{-/-} \) mice.
Fecal sterol loss independent of bile secretion

Abca1

Abcg5

Control
GW3965

A Wild-type

B \(Mdr2^{-/-}\)

- 207 kDa

- 94 kDa

Figure 4: Abca1 protein and Abcg5 protein expression in small intestines of wild-type and \(Mdr2^{-/-}\) mice maintained on chow diet in absence or presence of the LXR agonist GW3965.

Female wild-type FVB (A) and \(Mdr2^{-/-}\) (B) mice were maintained on chow diet in absence or present of the LXR agonist GW3965 (35 mg/kg/d) for 10 days. Homogenized small intestine samples (50 µg of total protein) were subjected to SDS/PAGE and immunoblotting for Abca1. Brush border membranes were isolated and subjected to SDS/PAGE and immunoblotting for Abcg5.

Plasma-derived cholesterol excretion in feces of \(Mdr2^{-/-}\) mice

In order to determine whether cholesterol excreted into the feces upon LXR activation could originate from the plasma compartment, untreated and GW3965-treated \(Mdr2^{-/-}\) mice received an intravenous injection of \([^{3}H]\)cholesterol dissolved in Intralipid. Although no difference could be detected in the specific activities of plasma cholesterol between both groups (page 116, Figure 5A), LXR activation did markedly increase (by 66%) the recovery of plasma-derived \([^{3}H]\)cholesterol in the neutral sterol fraction of the feces after 4 days by 66% (6.87 ± 0.55 vs. 4.13 ± 0.68 %/dose) (Figure 5B). As expected, no \([^{3}H]\) was detectable in the sterol fraction of bile after Bligh and Dyer extraction (data not shown).

Discussion

Reverse cholesterol transport (RCT) is usually defined as the pathway responsible for removal of excess cholesterol from peripheral cells by HDL, its transport to the liver and, finally, disposal from the body. LXR activation has been proposed to enhance RCT by induction of Abca1 (and other transporters) in peripheral cells to allow cholesterol efflux towards HDL, increasing biliary cholesterol secretion and, ultimately, stimulation of fecal cholesterol output. Recently, we calculated that part of the increased fecal cholesterol output upon LXR activation in mouse models must be of intestinal origin. These calculations were, however, based on the estimates of 24h biliary cholesterol secretion. In the present study, we therefore chose to dissect hepatobiliary cholesterol secretion from intestinal cholesterol secretion by using \(Mdr2^{-/-}\) mice, a model in which hepatobiliary cholesterol secretion is almost absent.
Figure 5: Specific activity of [$^3$H]cholesterol in plasma and cumulative excretion of radioactivity in the neutral sterol fraction of feces in Mdr2$^{-/-}$ mice treated with or without the synthetic LXR agonist GW3965.

Female individually housed Mdr2$^{-/-}$ mice were maintained on chow diet in the absence (n = 4) or presence (n = 5) of LXR agonist GW3965 (35 mg/kg/d) for 12 days. After 8 days of diet mice received [$^3$H]cholesterol intravenously and blood samples were taken and feces were collected every 24 hours until the end of the experiment. Radioactivity was measured in samples as described in MATERIALS AND METHODS. * indicate significant difference (Mann-Whitney U test, $p<0.05$).

As previously shown, LXR activation in wild-type mice led to increased HDL levels, increased biliary cholesterol secretion, decreased fractional cholesterol absorption and stimulation of fecal neutral sterol loss. In wild-type mice, but not in Mdr2$^{-/-}$ mice, LXR activation also led hepatic steatosis.\textsuperscript{21,24} Greffhorst et al. proposed that the development of hepatic steatosis upon LXR activation is related to increased de novo lipogenesis in combination with an increased free fatty acid flux toward the liver.\textsuperscript{21} The lack steatosis upon LXR activation in Mdr2$^{-/-}$ mice is under investigation.

As reported before,\textsuperscript{25} Mdr2$^{-/-}$ mice had reduced plasma HDL levels compared to wild-type mice. Expression of Abca1 appears to be a major determinant of plasma HDL.\textsuperscript{26,27} Our data show that, in Mdr2$^{-/-}$ mice, Abca1 mRNA expression was significantly reduced in the intestine as well as in the liver (data not shown). The reason herefore remains to be elucidated, but reduced Abca1 expression is a plausible explanation for low HDL levels in Mdr2$^{-/-}$ mice.
As expected, biliary cholesterol secretion in $Mdr2^{-/-}$ mice was severely impaired, i.e., by 87% compared to wild-type mice, whereas biliary phospholipid secretion was undetectable. Fecal neutral sterol and bile salt outputs were, however, similar to that observed in wild-type mice. These findings in female $Mdr2^{-/-}$ mice differ from previous observations in our laboratory, i.e., an increased fecal neutral sterol loss in male $Mdr2^{-/-}$ mice compared to sex- and age-matched controls. As this difference was rather striking, we additionally measured fecal neutral sterol loss in male wild-type and $Mdr2^{-/-}$ mice kept on the same diet as the female mice studied. From these analyses it appeared that fecal neutral sterol output, although two-fold higher compared to females, was similar in male wild-type and $Mdr2^{-/-}$ mice (data not shown). To date, we cannot explain this discrepancy in outcome of studies that were conducted 6 years apart from each other. We suggest that differences in age of the mice, housing conditions and, particularly, changes in chow composition may contribute to these divergent results.

Fractional cholesterol absorptions in untreated female $Mdr2^{-/-}$ mice was found to be similar to that of wild-type mice. This was surprising, as $Mdr2^{-/-}$ mice lack biliary phospholipids which is expected to limit incorporation of cholesterol into micelles. Cholesterol absorption measurements using the plasma dual-isotope method employing i.v. administration of $[^3H]$cholesterol and oral administration of $[^14C]$cholesterol in male by Voshol et al. indicated a decreased cholesterol absorption in $Mdr2^{-/-}$ mice. However, as already stated in the original paper, these results should be judged with caution as the liver and intestine did not reach in isotopical equilibrium with plasma within 48 hours. Clearly, intrinsic differences between both techniques to measure cholesterol absorption have to be taken in account. Data from the currently used test indicate that $Mdr2^{-/-}$ mice take up cholesterol normally from the lumen, as supported by unaffected $Npc1l1$ expression and the similar $[^14C]$cholesterol content of the intestinal wall after termination of the experiment. However, it is plausible to assume that the delayed chylomicron production in $Mdr2^{-/-}$ mice influences further processing of the enterocytic cholesterol pools.

Biliary cholesterol secretion in $Mdr2^{-/-}$ mice remained impaired upon LXR activation, demonstrating that phospholipid secretion is a prerequisite for hepatobiliary cholesterol excretion also during LXR-induced hypersecretion. Surprisingly, however, fecal neutral sterol output increased to a similar extent in wild-type and $Mdr2^{-/-}$ mice. As dietary intake was similar in all groups and biliary cholesterol was almost absent in $Mdr2^{-/-}$ mice, a considerable part of this cholesterol has to originate directly from the intestine.

To gain insight in the relative magnitude of LXR-induced “direct” intestinal secretion, it was of importance to assess the effects of LXR activation on fractional cholesterol absorption in both strains. As demonstrated by us and others, fractional cholesterol absorption was reduced by about 50% in wild-type mice upon LXR activation. Surprisingly, a much greater reduction was observed in the $Mdr2^{-/-}$ mice, indicating a virtually complete suppression of fractional cholesterol absorption upon treatment. The combination of a pronounced reduction...
of intestinal Npc1l1 expression, increased Abcg5/Abcg8 expression and relatively small amount of intraluminal cholesterol due to absence of biliary cholesterol are probably responsible for this surprising finding. Knowing the daily input of dietary cholesterol, as estimated from measurement of food intake, and biliary cholesterol, as calculated on the basis of 30 min output rates, and the fractional absorption rates, it is possible to estimate the apparent contribution of direct intestinal excretion to total fecal neutral sterol output. This calculated contribution was 20 % in untreated wild-type mice, which increased to 38 % upon LXR activation. Corresponding values for untreated and treated Mdr2-/- mice were 41% and 57%.

Part of this cholesterol could, in principle, originate from enhanced sloughing of intestinal cells or be due to increased de novo cholesterol synthesis. Based on unchanged HMGCoA reductase gene expression, we consider it unlikely that cholesterol synthesis was increased upon LXR activation. Staining for the proliferation marker Ki-67 revealed no signs of increased intestinal cell proliferation upon LXR activation, making the possibility of enhanced cell shedding less likely. We also showed that the specific activity of cholesterol in plasma remained unchanged upon LXR activation in Mdr2-/- mice, whereas the excretion of plasma-derived labeled cholesterol into feces was increased (Figure 5). The amount of radio-labeled fecal neutral secretion at 24 h after injection was about 0.7 % /dose in untreated Mdr2-/- mice. Assuming equilibration of the labeled cholesterol within 24 h\(^{32}\) and a total cholesterol pool of 143 \(\mu\)mol\(^{33}\) it can be calculated that about 1.0 \(\mu\)mol cholesterol leaves the body via this route per day. This value approximates 64% of the amount of neutral sterol actually measured in the stool of these mice, which is of the same order of magnitude as calculated from the fractional absorption rate. Since part of the labeled cholesterol may be reabsorbed this value may underestimate the true plasma-to-intestine flux. These three observations together strongly indicate that the intestine, independently of biliary cholesterol, plays an important role in the disposal of plasma-derived cholesterol from the body upon LXR activation. Therefore, we consider intestinal excretion a second route for cholesterol transport from the periphery to the feces and, by definition, an intrinsic part of the RCT system.

The question remains, however, which pathway mediates the uptake of plasma-derived cholesterol by the enterocyte. In addition to that of Abcg5 and Abcg8, intestinal gene expression of Abca1 and Abcg1 was increased upon LXR activation. Abca1 is known to be localized at the basolateral membranes of CaCo-2 cells\(^{34}\) and of chicken enterocytes\(^{35}\) and may facilitate uni-directional cholesterol transport from these cells towards HDL.\(^{36}\) Thus it is highly unlikely that Abca1 contributes to uptake of cholesterol into the enterocyte. Moreover, Abca1-/- mice increase their fecal cholesterol secretion – in the absence of HDL - upon LXR activation to the same extent as wild-type mice do.\(^3\) Abcg1 is known to regulate cholesterol and phospholipid transport in macrophages.\(^{37}\) Localization and function of Abcg1 in the intestine is currently unknown, but based on the macrophage study\(^{37}\) it is very likely that Abcg1 plays a role in intracellular lipid transport processes.
SRBI, at least in macrophages, has been implicated in bi-directional flux of cholesterol and phospholipids. The direction of cholesterol transport is probably determined by the free energy difference between cholesterol pools. In the small intestine, Sr-b1 is localized both at the apical membrane of the brush border and at the basolateral membrane, with different expression levels along the length of the small intestine. This suggests that Sr-b1 has several functions in the intestine. Mice deficient in Sr-b1, however, show only a small increase in cholesterol absorption efficiency and small decrease in fecal neutral sterol output. This suggests a relatively small contribution of intestinal Sr-b1 to control of fecal cholesterol excretion. However, basolaterally located Sr-b1 could theoretically play a role in cholesterol uptake into the enterocyte particularly when, due to activation of Abcg5 and Abcg8, free cholesterol in the enterocytes decreases and uptake of the sterol from the plasma compartment may become energetically favorable.

In conclusion, our results demonstrate that increased fecal neutral sterol excretion upon LXR activation is independent of biliary cholesterol excretion in mice. We propose the intestine as an additional excretory organ in RCT, implying that the "classical" definition of RCT needs to be redefined.

Acknowledgements:

We thank Julius F. W. Baller and Vincent W. Bloks for excellent technical assistance. Michael R. Hayden is thanked for generous supply of anti-Abca1 antibody. This study was supported by the Netherlands Heart Foundation (grant 2001B043).
Chapter 5

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