# The ABC of cholesterol transport

Plösch, Torsten

# CHAPTER 2

# Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X-receptor (LXR) is independent of ABCA1

Torsten Plösch[1]*
Tineke Kok[1]*
Vincent W. Bloks[1]
Martin J. Smit[2]
Rick Havinga[1]
Giovanna Chimini[3]
Albert K. Groen[4]
Folkert Kuipers[1]

[1] Center for Liver, Digestive, and Metabolic Diseases, Groningen University Institute for Drug Exploration, Department of Pediatrics, University Hospital Groningen, Groningen, The Netherlands
[2] Department of Pharmacology, Organon BV, Oss, The Netherlands
[3] Centre de Immunologie, INSERM-CNRS, Marseille, France
[4] Department of Gastroenterology, Academic Medical Center, Amsterdam, The Netherlands

* Equally contributed to this study

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## Summary

The ATP-binding cassette transporter Abca1 is essential for HDL formation and considered rate-controlling for reverse cholesterol transport. Expression of the *Abca1* gene is under control of the liver X-receptor (LXR). We have evaluated effects of LXR activation by the synthetic agonist T0901317 on hepatic and intestinal cholesterol metabolism in C57BL/6J and DBA/1 wild-type mice and in Abca1-deficient DBA/1 mice. In wild-type mice, T0901317 increased expression of *Abca1* in liver and intestine which was associated with a ~60% rise of HDL. Biliary cholesterol excretion rose 2.7-fold upon treatment and fecal neutral sterol output was increased by 150-300%. Plasma cholesterol levels also increased in treated *Abca1*$^{-/-}$ mice (+120%), but exclusively in VLDL-sized lipoprotein fractions. Despite the absence of HDL, hepatobiliary cholesterol output was stimulated upon LXR-activation in *Abca1*$^{-/-}$ mice, leading to 250% increase in biliary cholesterol:phospholipid ratio. Most importantly, fecal neutral sterol loss was induced to a similar extent (+300%) by the LXR agonist in DBA/1 wild-type and *Abca1*$^{-/-}$ mice. Expression of *Abcg5* and *Abcg8*, recently implicated in biliary excretion of cholesterol and its intestinal absorption, was induced in T0901317-treated mice. Thus, activation of LXR in mice leads to enhanced hepatobiliary cholesterol secretion and fecal neutral sterol loss independent from (Abca1-mediated) elevation of HDL and the presence of Abca1 in liver and intestine.

# Introduction

Reverse cholesterol transport (RCT) or centripetal cholesterol flux is a key process in maintenance of whole body cholesterol homeostasis.[1-6] RCT involves efflux of excess cholesterol from peripheral cells towards nascent HDL, its transport to the liver followed by hepatic uptake mediated by scavenger receptor class B type I (SR-BI), biliary secretion in the form of cholesterol or bile salt, and finally disposal into feces. HDL-mediated RCT is generally assumed to underlie the well-known epidemiological relationship between high HDL-cholesterol levels and low risk for development of atherosclerosis.

Efflux of cholesterol from peripheral cells, including macrophages in the vessel wall, is now known to be mediated in part by the ATP-binding cassette (ABC-) transporter Abca1.[7-10] *Abca1* mRNA is widely distributed throughout the body, with high expression levels in macrophages, hepatocytes, and enterocytes.[11,12] This distribution pattern has recently been confirmed for the Abca1 protein.[13] The role of Abca1 in hepatocytes is currently unknown but may involve formation of pre-β-HDL particles.[14] In the intestine, Abca1 has been suggested to be involved in cholesterol efflux from enterocytes into the lumen, thereby regulating the efficiency of intestinal cholesterol absorption. [15, 16]

HDL is considered a major source for bile-destined cholesterol and phospholipid.[17,18] Yet, we have recently demonstrated that, despite absence of HDL, hepatobiliary cholesterol flux and fecal sterol excretion are not affected in *Abca1* knockout mice.[19] Our results thus questioned whether Abca1 has indeed an important role in control of mass cholesterol transport from the periphery to the liver and suggest that its major peripheral function is removal of excess cholesterol from macrophages. Haghpassand *et al.*[20] showed convincingly that efflux from macrophages constitutes only a small fraction of HDL cholesterol.

Several genes involved in control of cholesterol metabolism, including *Abca1*, are transcriptionally regulated by the liver X-receptor (LXR).[21-24] Two LXR isoforms have been identified, LXRalpha (NR1H3) and LXRbeta (NR1H2).[25,26] Upon stimulation by oxysterols, activated LXR forms a heterodimer with the retinoid X-receptor (RXR, NR2B1), binds to DNA and influences gene expression. It has been proposed that a high dietary cholesterol intake, via subsequent formation of oxysterols, activates LXR which, in turn, induces expression of genes involved in cholesterol disposal.[27,28] Because of its prominent position in controlling cholesterol homeostasis, pharmacological activation of LXR is considered a promising approach to raise HDL, improve RCT and thereby prevent the development of atherosclerosis. Treatment of rodents with LXR (or RXR) agonists indeed results in elevation of plasma HDL levels[29,30] and reduced intestinal cholesterol absorption.[23]

In this study, we have investigated the role of Abca1 in LXR-controlled pathways of hepatobiliary and fecal cholesterol output in mice. For this purpose, wild-type mice and Abca1-deficient mice[31] were treated with the synthetic LXR agonist T0901317.[23,29] Surprisingly, both T0901317-treated *Abca1*$^{-/-}$ and wild-type mice showed similarly increased

rates of hepatobiliary cholesterol output and increased fecal sterol loss, independent from (Abca1-mediated) elevation of plasma HDL levels and the (putative) role of Abca1 in intestinal cholesterol absorption.

## Materials and Methods

### Animals

Male C57BL/6J mice, 2-3 months old, were purchased from Harlan (Horst, The Netherlands). *Abca1*$^{-/-}$ mice with a DBA/1 background, 6-8 months old, and age-matched DBA/1 wild-type mice were obtained from IFFA Credo (Saint-Germain-sur-L'Arbresle, France). Because of the limited supply of homozygous knockout mice, both male and female mice were used in these experiments. Animals received standard mouse chow (Hope Farms BV, Woerden, The Netherlands) and water *ad libitum*. Experimental procedures were approved by the local Ethical Committee for Animal Experiments.

### Experimental procedures

The synthetic LXR-agonist T0901317, kindly provided by Organon BV (Oss, The Netherlands), was solubilized in DMSO. This solution was diluted 1:1 with cremophor, and further diluted 1:9 with mannitol/water (5%). Animals received 20 μmol/kg T0901317 per day by gavage at 4 PM. Control groups were treated with the solvent only. All animals were housed separately and feces of individual mice were collected from day 4 to day 5. At day 5, mice were anaesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and Diazepam (10 mg/kg). Bile was collected for thirty minutes by cannulation of the gallbladder. During bile collection, body temperature was stabilized using an humidified incubator. At the end of the collection period, 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 PBS and divided into three equal parts. Parts of both 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 and Oil-red-O staining. C57BL/6J mice used for RNA isolation and lipid analysis only were sacrificed without prior bile collection. Tissues were immediately removed, snap-frozen in liquid nitrogen and manipulated as described below.

**Analytical procedures**

Bile salts were measured enzymatically.[32] Commercially available kits were used for the determination of free cholesterol (Wako, Neuss, Germany); total cholesterol, HDL-cholesterol, triglycerides (Roche, Mannheim, Germany); and phospholipids and free fatty acids (Wako) in plasma. Hepatic and biliary lipids were extracted according to Bligh and Dyer.[33] Phospholipids in bile and liver were determined as described by Böttcher *et al.*[34] Cholesterol in bile was measured according to Gamble *et al.*[35] Hepatic cholesterol and triglyceride contents were analyzed as described above. Feces were lyophilized, weighed and homogenized. Neutral sterols and bile salts were analyzed according to Arca *et al.*[36] and Setchell *et al*.,[37] respectively. Pooled plasma samples from all animals of one group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) as described previously.[38]

**RNA isolation and PCR procedures**

Total RNA was isolated with Trizol (Invitrogen, Carlsbad, USA) and quantified using Ribogreen (Molecular Probes, Eugene, USA). cDNA synthesis was done according to Bloks *et al.*[39] For C57BL/6J mice, all three intestinal samples per mouse were analyzed separately, whereas for DBA/1 and *Abca1*$^{-/-}$ mice equal amounts of RNA from the three distinct parts of the small intestine were pooled prior to reverse transcription. Real-time quantitative PCR[40] 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): all sequences are listed in Table I (page 46). All expression data were subsequently standardized for 18S ribosomal RNA which was analyzed in separate runs.

**Statistics**

Statistical analyses were performed using SPSS version 10.0 for Windows (SPSS Inc., Chicago, USA). Treated and untreated groups were compared by Student's *t*-test for large data series of biochemical parameters and by Mann-Whitney-U-test for the remaining, as indicated. A $p$-value$< 0.05$ was considered statistically significant.

**Table I:** Primer sequences used in mRNA quantification by real-time RT-PCR.

| | accession number | forward | reverse | probe |
|---|---|---|---|---|
| Srebp-1a | (ref. 59) | GAGGCGGCTCTGGAACAGA | TGTCTTCGATGTCGTTCAAAACC | TGTGTCCAGTTCGCACATCTCGGC |
| Srebp1-c | BI656094 | GGAGCCATGGATTGCACATT | CCTGTCTCACCCCCAGCATA | CAGCTCATCAACAACCAAGACAGTGACTTCC |
| Srebp-2 | AF374267 | CTGCAGCCTCAAGTGCAAAG | CAGTGTGCCATTGGCTGTCT | CCATCCAGCAGCAGGTGCAGACG |
| LXRalpha (NR1H3) | AF085745 | GCTCTGCTCATTGCCATCAG | TGTTGCAGCCTCTCTACTTGGA | TCTGCAGACCGGCCCAACGTG |
| HMG-CoA reductase | BB664708 | CCGGCAACAACAAGATCTGTG | ATGTACAGGATGGCGATGCA | TGTCGCTGCTCAGCACGTCCTCTTC |
| Cyp7a1 | NM_007824 | CAGGGAGATGCTCTGTGTTCA | AGGCATACATCCCTTCCGTGA | TGCAAAACCTCCAATCTGTCATGAGACCTCC |
| Cyp27 | AK004977 | GCCTTGCACAAGGAAGTGACT | CGCAGGGTCTCCTTAATCACA | CCCTTCGGGAAGGTGCCCCAG |
| Acat1 | NM_009230 | TGGGTGCCACTTCGATGACT | TGAGTGCACACCCACCATTG | CCAACCTCATTGAAAAGTCCGCATCGC |
| Acat2 | NM_011433 | GGTGGAACTATGTGGCCAAGA | CCAGGATGAAGCAGGCATAGA | CAAACAGCCCAGGACCTGGGCAAAG |
| Lpl | NM_008509 | AAGGTCAGAGCCAAGAGAAGCA | CCAGAAAAGTGAATCTTGACTTGGT | CCTGAAGACTCGCTCTCAGATGCCCTACA |
| Abca1 | NM_013454 | CCCAGAGCAAAAAGCGACTC | GGTCATCATCACTTTGGTCCTTG | AGACTACTCTGTCTCTCAGACAACACTTGACCAAG |
| Abcg5 | AF312713 | TCAGGACCCCAAGGTCATGAT | AGGCTGGTGGATGGTGACAAT | CCACAGGACTGGACTGCATGACTGCA |
| Abcg8 | AK004871 | GACAGCTTCACAGCCCACAA | GCCTGAAGATGTCAGAGCGA | CTGGTGCTCATCTCCCTCCACCAG |
| Bsep (Abcb11) | NM_021022 | CTGCCAAGGATGCTAATGCA | CGATGGCTACCCTTTGCTTCT | TGCCACAGCAATTTGACACCCTAGTTGG |
| Mdr2 (Abcb4) | NM_008830 | GCAGCGAGAAACGGAACAG | GGTTGCTGATGCTGCCTAGTT | AAAGTCGCCGTCTAGGCGCCGT |
| Ntcp (Slc10a1) | AB003303 | ATGACCACCTGCTCCAGCTT | GCCTTTGTAGGGCACCTTGT | CCTTGGGCATGATGCCTCTCCTC |
| Oatp1 (Slc21a1) | NM_013797 | CAGTCTTACGAGTGTGCTCCAGAT | ATGAGGAATACTGCCTCTGAAGTG | TGGATTTGCCAGTACATTTACCTTCTTGCCC |
| SR-BI | NM_016741 | TCAGAAGCTGTTCTTGGTCTGAAC | GTTCATGGGGATCCCAGTGA | ACCCAAAGGAGCATTCCTTGTTCCTAGACA |
| 18S rRNA | X00686 | CGGCTACCACATCCAAGGA | CCAATTACAGGGCCTCGAAA | CGCGCAAATTACCCACTCCCGA |

All probes are labeled with FAM (6-carboxy-fluorescein) and TAMRA (6-carboxy-tetramethyl-rhodamine) at the 5'- and 3'-end, respectively.

# Results

### LXR activation by T0901317 increases plasma HDL and induces hepatic steatosis in C57BL/6J mice

Treatment with the LXR agonist T0901317 resulted in profound changes in plasma and liver lipid homeostasis in C57BL/6J mice, as previously reported by ourselves[41] and others.[23,29] On the treatment protocol employed in the current study, mice developed significantly elevated plasma levels of cholesterol, particularly in the esterified fraction, and phospholipids. HDL cholesterol was elevated by 59% upon treatment. Administration of T0901317 increased liver weight by 40% without any change in body weight. Hepatic total cholesterol content was decreased in treated mice (-15%), partly as a result of a significantly diminished cholesteryl-ester concentration (-32%), whereas the concentration of phospholipids was not affected. In treated animals, we found a more than 8-fold increase in hepatic triglyceride content, in accordance with recently published studies.[30] Histologically, these animals presented with profound hepatic fat deposits but no signs of liver damage were noticed (data not shown).

### LXR activation by T0901317 induces biliary hypersecretion of cholesterol in C57BL/6J mice

Bile flow was unaffected by T0901317 treatment when calculated on the basis of body weight (Table II). Biliary cholesterol output was 2.7-fold higher upon treatment, while biliary bile salt and phospholipid outputs were not affected. As a consequence, the ratio cholesterol/phospholipids increased from 0.07 to 0.23 upon treatment, indicative for uncoupling of biliary cholesterol from phospholipid secretion.

**Table II:** Bile flow and biliary secretion rates of C57BL/6J mice treated with the LXR agonist T0901317 or its solvent.

| | control | T0901317 |
|---|---|---|
| bile flow (μl/min/100 g body weight) | 8.2 ± 2.8 | 7.8 ± 2.6 |
| bile salts (nmol/min/100 g body weight) | 584 ± 229 | 477 ± 200 |
| cholesterol (nmol/min/100 g body weight) | 3.8 ± 1.4 | 10.3 ± 3.1* |
| phospholipids (nmol/min/100 g body weight) | 52.7 ± 10.8 | 44.2 ± 9.7 |
| ratio cholesterol/phospholipids | 0.07 ± 0.03 | 0.23 ± 0.04** |

Male C57BL/6J mice, 2-3 months old, were treated with the LXR agonist T0901317 or solvent only as described in MATERIALS AND METHODS; n=6 per group. Bile was collected for 30 minutes. Values represent means ± SD. * indicates significant difference (Mann-Whitney-U-test, $p<0.05$); ** indicates significant difference (Mann-Whitney-U-test, $p<0.001$).

Gene expression profiles of key regulatory, metabolic and transporter encoding genes involved in hepatic cholesterol metabolism were analyzed by real-time PCR (Table III). As expected,[42,43] the gene encoding for sterol regulatory element-binding protein 1c (*Srebp-1c)* was the only regulatory gene with a modified expression (2.6 fold up) upon T0901317 treatment. This predicted increase is indicative for the overall stimulatory action of the agonist on hepatic gene expression, also supported by ~5 fold increase in expression levels of the LXR-target genes *Lpl* encoding lipoprotein lipase (not shown). The gene encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*), the key enzyme in cholesterol synthesis, was upregulated by 55%, while the 45% upregulation of the bile salt synthesis gene *Cyp7a1* did not reach statistical significance. T0901317 treatment increased expression of *Abca1* and *Abcg5* 2.4-fold and 2.8-fold, respectively; hepatic *Abcg8 expression* showed a high variation in its expression levels. Expression of transporters involved in bile salt uptake (*Ntcp, Oatp1*) and secretion (*Bsep*) and in phospholipid secretion (*Mdr2*) remained unaffected.

**Table III:** mRNA expression levels in liver tissue of C57BL/6J mice treated with the LXR agonist T0901317 or its solvent measured by real-time RT-PCR.

| mRNA | control | T0901317 |
|---|---|---|
| Srebp-1a | 1.00 ± 0.18 | 1.22 ± 0.06 |
| Srebp-1c | 1.00 ± 0.15 | 2.64 ± 0.54* |
| Srebp-2 | 1.00 ± 0.17 | 1.00 ± 0.06 |
| LXR | 1.00 ± 0.09 | 0.84 ± 0.11 |
| HMG-CoA reductase | 1.00 ± 0.10 | 1.55 ± 0.36* |
| Cyp7a1 | 1.00 ± 0.42 | 1.45 ± 0.74 |
| Cyp27 | 1.00 ± 0.16 | 0.94 ± 0.08 |
| Acat2 | 1.00 ± 0.12 | 1.24 ± 0.27 |
| Abca1 | 1.00 ± 0.55 | 2.38 ± 0.96* |
| Abcg5 | 1.00 ± 0.42 | 2.81 ± 1.19* |
| Abcg8 | 1.00 ± 0.56 | 1.54 ± 0.51 |
| Bsep | 1.00 ± 0.18 | 1.01 ± 0.06 |
| Mdr2 | 1.00 ± 0.10 | 1.13 ± 0.17 |
| Ntcp | 1.00 ± 0.04 | 0.97 ± 0.10 |
| Oatp1 | 1.00 ± 0.41 | 0.63 ± 0.09 |

Male C57BL/6J mice, 2-3 months old, were treated with the LXR agonist T0901317 or solvent only as described in MATERIALS AND METHODS; n=4 per group. Quantitative real-time PCR was performed as described in MATERIALS AND METHODS with primers and probes given in Table I. All data were standardized for 18S ribosomal RNA. Expression in control mice was set to 1.00. Values represent means ± SD. * indicates significant difference (Mann-Whitney-U-test, $p<0.05$).

**LXR activation by T0901317 accelerates fecal sterol loss in C57BL/6J mice**

Fecal bile salt loss was increased by 84% upon activation of LXR with T0901317, reflecting increased hepatic bile salt synthesis (Figure 1). In addition, neutral sterol output was enhanced by 187% in T0901317-treated mice. Increased expression of *Abca1* in the intestine has been proposed to reduce the efficacy of cholesterol (re-)absorption and hence enhance fecal cholesterol disposal.[23] Indeed, treatment of mice with the LXR agonist T0901317 resulted in an approximately 3-fold increase in *Abca1* mRNA abundance along the entire length of the small intestinal tract (Figure 2A). Likewise, the expression of *Abcg5* and *Abcg8*, recently implicated in control of cholesterol absorption,[44-46] was induced in treated animals albeit less pronounced than that of *Abca1* (Figures 2B and 2C). In contrast, mRNA levels of *Hmgcr* and *Acat1* (encoding for acyl-coenzyme A:cholesterol acyltransferase 1), indicative for intestinal cholesterol synthesis and cholesterol esterification, respectively, were similar in treated and control animals (Figure 2D, E). No changes in intestinal morphology were noted upon microscopical examination of hematoxylin/eosin and Oil-red-O stained sections (data not shown).

To elucidate the specific role of Abca1 in the observed LXR-mediated stimulation of cholesterol disposal in mice, we subsequently conducted a series of similar experiments in *Abca1*$^{-/-}$ mice and adequate wild-type controls on a DBA/1 background. Both male and female mice were used in these studies: no specific gender effects on the parameters studied were noted unless otherwise stated. Therefore, outcome is in most cases presented as average values per group.

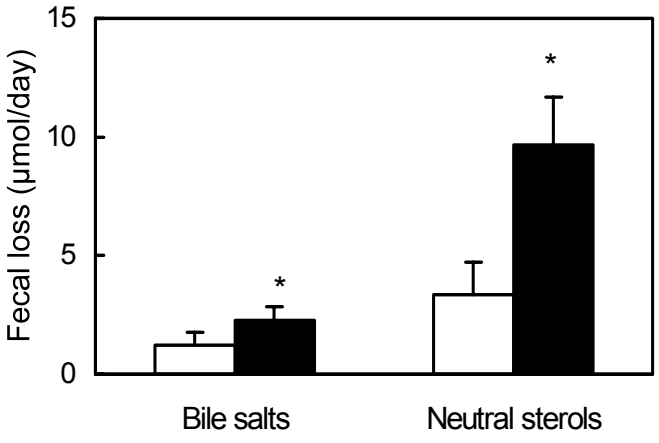


**Figure 1:** Fecal loss of neutral sterols and bile salts of C57BL/6J mice treated with the LXR agonist T0901317 or its solvent.

C57BL/6J mice were treated with T0901317 (filled bars) or solvent only (open bars) for four days (n=10 per group); feces were collected during the last 24 hours of the experiment and analyzed as described in MATERIALS AND METHODS. * indicates significant difference (t-test, $p<0.001$).

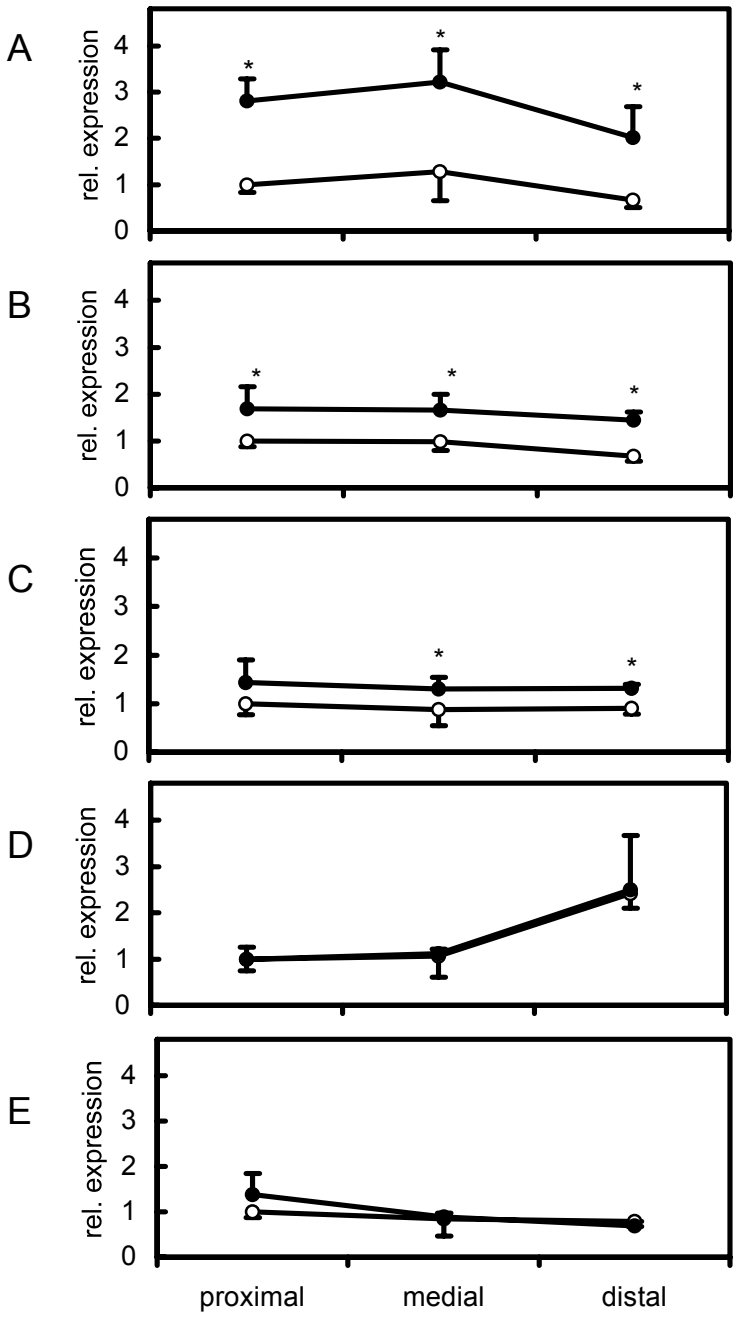


**Figure 2**: mRNA expression levels in the intestine of C57BL/6J mice treated with the LXR agonist T0901317 or its solvent measured by real-time PCR.

C57BL/6J mice were treated with T0901317 (filled circles) or solvent only (open circles) for four days (n=4 per group); the intestine was removed, rinsed with cold PBS, divided into three equal parts and analyzed as described in MATERIALS AND METHODS. All data were standardized for *18S ribosomal RNA*. Expression in the proximal part of the small intestine in animals receiving the solvent only was set to 1. * indicates significant difference (Mann-Whitney-U-test, $p<0.05$).

A, relative expression of *Abca1*.

B, relative expression of *Abcg5*.

C, relative expression of *Abcg8*.

D, relative expression of *Hmgcr*.

E, relative expression of *Acat1*.

**LXR activation by T0901317 increases cholesterol in VLDL-sized lipoprotein fractions in *Abca1*$^{-/-}$ mice**

Upon treatment with T0901317, plasma concentrations of free cholesterol were increased in both *Abca1*$^{-/-}$ and wild-type DBA/1 mice (Table IV). Cholesterylester concentrations were not significantly affected, resulting in an increase in total cholesterol in *Abca1*$^{-/-}$ mice only. Both plasma phospholipid and triglyceride levels were not significantly changed upon T0901317-mediated activation of LXR in DBA/1 mice. FPLC-separation of plasma lipoproteins revealed that, as anticipated, the increase in plasma cholesterol in wild-type mice was in the HDL-sized

lipoprotein fraction. Figure 3 illustrates the lipoprotein pattern in T0901317-treated wild-type and *Abca1*$^{-/-}$ mice. Upon LXR activation, wild-type mice showed elevated cholesterol concentrations in the HDL-sized fractions, whereas *Abca1*$^{-/-}$ mice showed increased cholesterol concentrations in the VLDL-sized fractions.

**Table IV:** Plasma lipid levels in wild-type DBA/1 and *Abca1*$^{-/-}$ mice treated with the LXR agonist T0901317 or its solvent.

| | wild-type | | *Abca1*$^{-/-}$ | |
|---|---|---|---|---|
| | control | T0901317 | control | T0901317 |
| total cholesterol (mM) | 1.12 ± 0.55 | 1.64 ± 0.69 | 0.50 ± 0.30 | 1.11 ± 0.33* |
| free cholesterol (mM) | 0.35 ± 0.14 | 0.62 ± 0.10* | 0.29 ± 0.08 | 0.72 ± 0.25* |
| cholesterylester (mM) | 0.77 ± 0.42 | 1.02 ± 0.69 | 0.21 ± 0.28 | 0.39 ± 0.14 |
| phospholipids (mM) | 1.26 ± 0.61 | 2.07 ± 1.28 | 0.75 ± 0.43 | 1.34 ± 0.28 |
| triglycerides (mM) | 0.73 ± 0.33 | 0.93 ± 0.28 | 0.83 ± 0.38 | 1.88 ± 1.39 |

Male and female wild-type DBA/1 and *Abca1*$^{-/-}$ mice, 6-8 months old, were treated with the LXR agonist T0901317 or solvent only as described in MATERIALS AND METHODS; n=5-6 per group. Blood was collected by cardiac puncture. Values represent means ± SD. * indicates significant difference (Mann-Whitney-U-test, $p<0.05$).

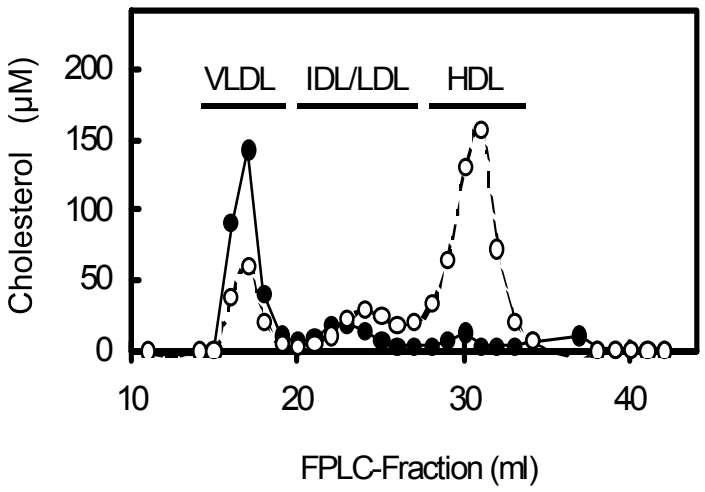


**Figure 3:** FPLC analysis of plasma cholesterol of DBA/1 and *Abca1*$^{-/-}$ mice treated with the LXR agonist T0901317 or its solvent.

DBA/1 wild-type mice (open circles, dotted line) and *Abca1*$^{-/-}$ (closed circles, full line) mice were treated with T0901317 for four days (n=5 per group). Blood was collected via cardiac puncture and pooled before FPLC analysis. Analysis was performed as described in MATERIALS AND METHODS.

**Table V:** Bile flow and biliary secretion rates in wild-type DBA/1 and *Abca1*$^{-/-}$ mice treated with the LXR agonist T0901317 or its solvent.

| | wild-type | | *Abca1*$^{-/-}$ | |
|---|---|---|---|---|
| | control | T0901317 | control | T0901317 |
| bile flow (μl/min/100 g body weight) | 7.2 ± 0.9 | 7.4 ± 1.2 | 7.8 ± 1.6 | 7.9 ± 1.8 |
| bile salts (nmol/min/100 g body weight) | 392 ± 68 | 307 ± 119 | 472 ± 213 | 251 ± 120* |
| cholesterol (nmol/min/100 g body weight) | 8.0 ± 1.1 | 17.0 ± 3.2* | 8.4 ± 2.5 | 19.5 ± 7.5* |
| phospholipids (nmol/min/100 g body weight) | 63.1 ± 7.0 | 38.1 ± 7.7* | 67.6 ± 16.2 | 48.6 ± 22.3 |
| ratio cholesterol/phospholipids | 0.13 ± 0.02 | 0.46 ± 0.13* | 0.12 ± 0.03 | 0.42 ± 0.08* |

Male and female wild-type DBA/1 and *Abca1*$^{-/-}$ mice, 6-8 months old, were treated with the LXR agonist T0901317 or solvent only as described in MATERIALS AND METHODS; n=5-6 per group. Bile was cannulated for 30 minutes. Values represent means ± SD. * indicates significant difference (Mann-Whitney-U-test, $p<0.05$).

**Table VI:** mRNA expression levels in liver tissue of wild-type DBA/1 and *Abca1*$^{-/-}$ mice treated with the LXR agonist T0901317 or its solvent measured by real-time RT-PCR.

| | wild-type | | *Abca1*$^{-/-}$ | |
|---|---|---|---|---|
| mRNA | control | T0901317 | control | T0901317 |
| Srebp-1c | 1.00 ± 0.31 | 2.04 ± 1.22 | 1.17 ± 0.49 | 2.49 ± 0.78* |
| HMG-CoA reductase | 1.00 ± 0.25 | 0.90 ± 0.36 | 0.60 ± 0.22 | 1.18 ± 0.43* |
| Cyp7a1 | 1.00 ± 0.36 | 0.60 ± 0.26 | 0.92 ± 0.40 | 0.46 ± 0.20 |
| SR-BI | 1.00 ± 0.14 | 0.73 ± 0.19* | 0.81 ± 0.20 | 0.69 ± 0.12 |
| Abca1 | 1.00 ± 0.41 | 0.80 ± 0.38 | 0.53 ± 0.17 | 0.52 ± 0.08 |
| Abcg5 | 1.00 ± 0.14 | 1.75 ± 0.85 | 0.75 ± 0.28 | 1.91 ± 0.67* |
| Abcg8 | 1.00 ± 0.16 | 1.65 ± 0.68 | 0.81 ± 0.16 | 1.51 ± 0.48* |
| Bsep | 1.00 ± 0.14 | 1.07 ± 0.45 | 0.86 ± 0.16 | 1.02 ± 0.40 |
| Mdr2 | 1.00 ± 0.27 | 0.83 ± 0.21 | 0.88 ± 0.16 | 0.76 ± 0.14 |

Male and female wild-type DBA/1 and *Abca1*$^{-/-}$ mice, 6-8 months old, were treated with the LXR agonist T0901317 or solvent only as described in MATERIALS AND METHODS; n=5 per group. cDNA synthesis and real-time PCR were performed as described in MATERIALS AND METHODS with primers and probes given in Table I. All data were standardized for *18S ribosomal RNA*. Expression in wild-type mice receiving solvent only was set to 1.00. Values represent means ± SD. * indicates significant difference (Mann-Whitney-U-test, $p<0.05$).

**Hepatobiliary cholesterol secretion is increased upon T0901317-treatment both in wild-type and *Abca1*$^{-/-}$ mice**

As shown in Table V, treatment with T0901317 did not change bile flow in wild-type or *Abca1*$^{-/-}$ mice. Bile salt secretion remained unchanged in wild-type mice but slightly decreased in T0901317-treated *Abca1*$^{-/-}$ mice. Phospholipid output rates were not altered in *Abca1*$^{-/-}$ mice and moderately lowered in wild-type mice upon LXR-activation. Expression of genes involved in hepatic cholesterol metabolism and of ABC-transporters known to be involved in bile formation, *i.e.* of *Bsep* and of *Mdr2* were not affected by Abca1-deficiency or LXR activation (Table VI). Biliary cholesterol secretion was markedly increased in T0901317-treated DBA/1 mice and, in spite of the absence of HDL, to the same extent in treated *Abca1*$^{-/-}$ mice. In both strains a more than 3 times higher cholesterol/phospholipid ratio was found in bile, indicative for cholesterol hypersecretion induced by LXR activation that is independent of Abca1 functioning.

**Fecal neutral sterol excretion is stimulated by LXR activation in the absence of Abca1**

Fecal sterol loss during the final 24 hours of the experiment is shown in Figure 4. Fecal bile salt output was similar in DBA/1 wild-type mice and *Abca1*$^{-/-}$ mice both under control conditions and upon LXR stimulation (Figure 4, upper panel). As expected, neutral sterol loss in

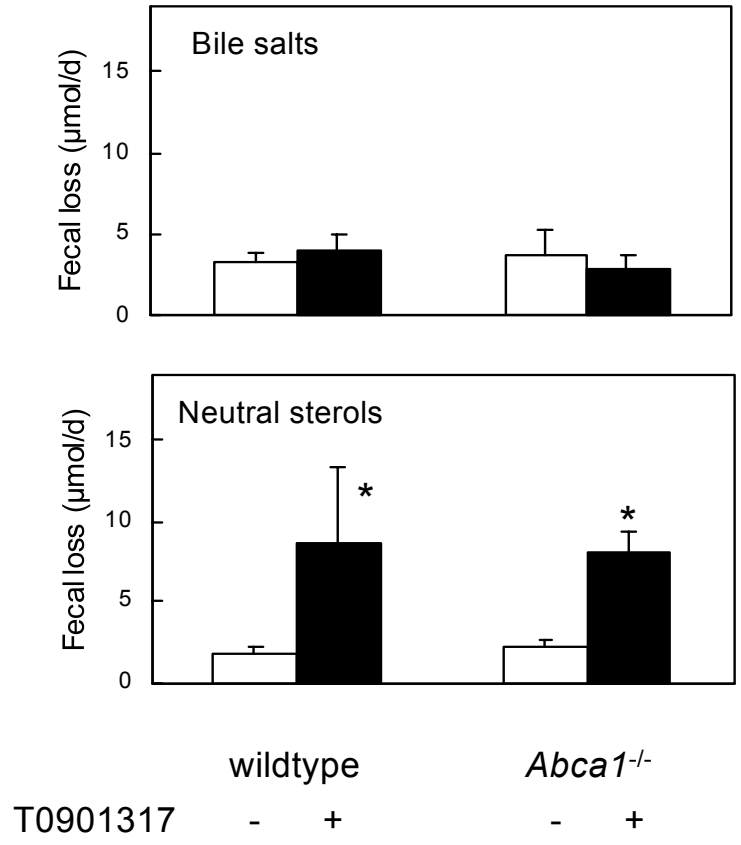


**Figure 4:** Fecal loss of neutral sterols and bile salts of DBA/1 and *Abca1*$^{-/-}$ mice treated with the LXR agonist T0901317 or its solvent.

Wild-type DBA/1 mice and *Abca1*$^{-/-}$ mice were treated with T0901317 (filled bars) or solvent only (open bars) for four days (n=5 per group). Feces were collected for the last 24 hours of the experiment and analyzed as described in MATERIALS AND METHODS. * indicates significant difference (Mann-Whitney-U-test, $p<0.05$).

wild-type mice was dramatically increased upon treatment (Figure 4, lower panel). Surprisingly, *Abca1*$^{-/-}$ mice showed a very similar response upon LXR activation, *i.e.,* a significantly higher neutral sterol output.

The entry of cholesterol into the intestinal lumen consists of at least three components: delivery via the bile, dietary intake, and direct intestinal secretion by enterocytes into the lumen, including shedding of cells. The rate of fecal cholesterol excretion (loss) is determined by the cumulative rate of cholesterol entry and the rate of (re)absorption of lumenal cholesterol. From earlier studies[38] we know that dietary intake is about 4 µmol/100g/day, which, under basal conditions, is similar to the biliary cholesterol flux in C57BL/6J mice. Basal rate of fecal cholesterol excretion in C57BL/6J mice is ~17 µmol/100g/day, which is higher than the sum of dietary and biliary cholesterol influx into the intestine. By inference, at least ~50% of fecal cholesterol must originate from the intestine. Assuming no major effect of T0901317-treatment on dietary cholesterol intake, the treatment did not affect the relative contribution of cholesterol from the intestine to the amount of cholesterol excretion via the feces, again at least ~50%. This implies, however, that T0901317-treatment significantly increased the absolute amount of cholesterol in the feces originating from the intestine. In DBA/1 wild-type

**Table VII:** mRNA expression levels in intestine of wild-type DBA/1 and *Abca1*$^{-/-}$ mice treated with the LXR agonist T0901317 or its solvent measured by real-time RT-PCR.

| | wild-type | | *Abca1*$^{-/-}$ | |
|---|---|---|---|---|
| mRNA | control | T0901317 | control | T0901317 |
| HMG-CoA reductase | 1.00 ± 0.13 | 0.98 ± 0.16 | 1.12 ± 0.30 | 0.87 ± 0.24 |
| Acat1 | 1.00 ± 0.18 | 1.84 ± 0.49* | 1.19 ± 0.61 | 1.47 ± 0.55 |
| Acat2 | 1.00 ± 0.19 | 1.14 ± 0.29 | 1.21 ± 0.60 | 1.01 ± 0.22 |
| Abca1 | 1.00 ± 0.35 | 2.59 ± 1.16* | 0.42 ± 0.12 | 1.26 ± 0.40* |
| Abcg5 | 1.00 ± 0.09 | 2.02 ± 0.31* | 0.94 ± 0.16 | 1.72 ± 0.48* |
| Abcg8 | 1.00 ± 0.12 | 1.84 ± 0.33 | 1.08 ± 0.33 | 1.90 ± 0.53* |
| SR-BI | 1.00 ± 0.20 | 2.31 ± 0.89* | 1.47 ± 0.57 | 1.76 ± 0.47 |

Male and female wild-type DBA/1 and *Abca1*$^{-/-}$ mice, 6-8 months old, were treated with the LXR agonist T0901317 or solvent only as described in MATERIALS AND METHODS; n=5 per group. RNA was isolated from three parts of the small intestine; equal amounts of RNA were pooled for cDNA synthesis and analysed as described in MATERIALS AND METHODS with primers and probes given in Table I. All data were standardized for *18S ribosomal RNA*. Expression in wild-type mice receiving solvent only was set to 1.00. Values represent means ± SD. * indicates significant difference (Mann-Whitney-U-test, $p<0.05$). For *Abca1*, about 40% remaining, putatively inactive, mRNA was detectable in *Abca1*$^{-/-}$ mice as the PCR-primers are located outside the deleted exons.[11,31]

and *Abca1*$^{-/-}$ mice under basal conditions, the sums of (assumed) dietary and biliary cholesterol influx into the intestine were higher than the fecal rate of cholesterol excretion, indicating net cholesterol absorption by the intestine. After T0901317-treatment, however, the sum of biliary and (assumed) dietary cholesterol influx into the intestine was *lower* than the fecal rate of cholesterol excretion, implying that the net intestinal cholesterol flux had shifted from absorption from the lumen to excretion into the lumen (data not shown).

Absence of any effect of genotype or treatment on intestinal expression of *Hmgcr* indicates that changes in fecal neutral sterol excretion are unlikely due to effects on intestinal cholesterol synthesis (Table VII). Expression of *Abca1* was clearly induced in the intestine of wild-type mice after treatment with T0901317. The intestinal expression of *Abcg5* and *Abcg8* was similar in wild-type and *Abca1*$^{-/-}$ mice and, in both strains, upregulated upon LXR activation. Due to high variation, there was no significant increase in *Abcg5* mRNA levels in the wild-type mice. This high variation was, at least partly, caused by less pronounced induction in male animals. In wild-type mice, expression of *Acat1* and *SR-BI* also increased significantly. This effect was less pronounced in *Abca1*$^{-/-}$ mice.

# Discussion

Activation of the nuclear receptor LXR influences multiple steps involved in maintenance of cholesterol homeostasis, particularly by inducing the expression of genes that control key steps in removal of excess cholesterol from the body. It has been postulated [15,16] that LXR agonists may combine 3 potentially anti-atherogenic effects, *i.e.*, increased efflux from peripheral tissues towards HDL by upregulation of *Abca1* and *Abcg1*, increased catabolism of cholesterol by upregulation of bile salt synthesis, and inhibition of dietary cholesterol absorption via upregulation of intestinal cholesterol transporters like Abca1, Abcg5 and Abcg8. The current study demonstrates that short-term administration of the LXR agonist T0901317 elevates plasma HDL levels in wild-type mice, both of C57BL/6J and DBA/1 backgrounds, as previously described.[29,30] This is accompanied by a marked hypersecretion of cholesterol into bile and a strongly increased fecal excretion of neutral sterols. Biliary secretion of phospholipids and, in the wild-type mice, also of bile salts remained largely unaffected. Hence, LXR activation fully uncoupled cholesterol from other biliary lipids. Surprisingly, increased hepatobiliary and fecal cholesterol disposal was found to be totally independent from Abca1-mediated HDL formation and the (putative) contribution of Abca1 in the control of intestinal cholesterol absorption, since the effects of LXR activation on these parameters were indistinguishable between DBA/1 wild-type and *Abca1*$^{-/-}$ mice. Moreover, the results of this study strongly indicate that pharmacological LXR activation stimulates direct efflux of cholesterol from the intestinal epithelium into the lumen. Based on the assumption that dietary intake of cholesterol was identical in all groups and an estimate of 24 hours biliary cholesterol

excretion, this efflux was calculated as the difference between dietary and biliary input minus fecal output. LXR activation greatly increased this flux. From our data, however, we cannot draw conclusions concerning the relative contributions of decreased absorption, increased direct sterol excretion by intestinal cells and accelerated shedding of enterocytes to this net loss of sterols. There were no indications for a compensatory increase in intestinal cholesterol synthesis, since *Hmgcr* expression remained unaffected along the length of the small intestine. Independent of the mechanism, this finding delineates the important role of the intestine in cholesterol homeostasis, as suggested by us previously,[19,38,47] and that the presence of Abca1 is not required to fulfill this role. In line with the proposed role of the Abc half transporters Abcg5 and Abcg8 in cholesterol efflux towards the intestinal lumen[44,45] and the consistently induced expression of these genes in intestines of LXR-agonist-treated mice, it is tempting to speculate that these half transporters have a crucial role in LXR-induced changes in intestinal cholesterol metabolism.

The classical view of reverse cholesterol transport[1] predicts that, under steady state conditions, all cholesterol synthesized in peripheral organs is eventually transported by HDL to the liver for excretion into bile followed by its disposal via the feces. Abca1 is considered a crucial factor in this process, since absence of a functional protein in Tangier Disease[7-9] and in *Abca1*$^{-/-}$ mice[31] is associated with a complete lack of HDL. The validity of the reverse cholesterol transport concept to explain mass cholesterol flux from periphery to liver has been questioned by series of studies in mice showing that the magnitude of RCT (or "centripetal cholesterol flux") is not determined by plasma HDL levels[48-53] and not affected by stimulation of individual key steps in the process, such as cholesterol efflux from peripheral tissues, SR-BI-mediated uptake of HDL cholesterol by the liver and conversion of cholesterol into bile salts.[54] In addition, we have recently shown that the absence of HDL due to Abca1-deficiency does not alter hepatic cholesterol synthesis, which would be anticipated if HDL accommodates a quantitative important cholesterol flux towards the liver, nor does it affect biliary cholesterol excretion and fecal sterol loss.[19] These observations, together with the fact that macrophages contribute only modestly to HDL cholesterol,[20] indicate that the relevance of Abca1 and of high HDL in protection from atherosclerosis is related to events at the level of the vessel wall rather than to stimulation of mass cholesterol flux. This, in turn, implies that the absence of HDL in patients with Tangier disease and *Abca1*$^{-/-}$ mice is not due to the absence of the protein in macrophages. Since specific overexpression of Abca1 in hepatocytes and macrophages of *Abca1*$^{-/-}$ mice by using an adenoviral construct driven by the *Apoe* promoter normalizes plasma HDL,[14] it is tempting to speculate that hepatic Abca1 has a role in formation of nascent HDL particles. Thus, it is highly likely that elevated HDL levels in T0901317-treated mice are attributable to increased hepatic Abca1 expression and function. Elevated VLDL-cholesterol in treated *Abca1*$^{-/-}$ mice may be due to enhanced formation of VLDL particles by the liver. Recent data from our laboratory show that LXR activation strongly promotes the production of large, triglyceride-rich VLDL particles by the liver in

wild-type mice.[41] Whether or not there are qualitative or quantitative differences in this respect between wild-type and *Abca1*$^{-/-}$ mice remains to be established.

There are data to indicate that HDL-cholesterol is an important source for both biliary cholesterol and for bile salt synthesis.[17,18] The similar increase in biliary cholesterol excretion in *Abca1*$^{-/-}$ mice and their controls upon LXR activation shows, at least, that other sources are able to fully compensate for the lack of HDL cholesterol in *Abca1*$^{-/-}$ mice. As hepatic cholesterylester concentrations were diminished in treated mice independently of presence of Abca1 by ~50-85%, part of excess biliary cholesterol may have been derived from hepatic stores. Expression of *Hmgcr* was slightly increased in T0901317-treated mice, indicating that a compensatory increase in synthesis also may contribute. In spite of the fact that *Cyp7a1* has been identified as a *bona fide* LXR target gene *in vitro*,[55] we observed only a modest (C57BL/6J mice) or no (DBA/1 mice) increase in fecal bile salt secretion upon LXR activation, indicating limited effects on total bile salt synthesis. Accordingly, no significant effects on *Cyp7a1* expression levels were found. The fact that, in contrast to other reports,[23,29,43] we did not find an induction of *Cyp7a1* expression upon T0901317-administration, is probably related to differences in treatment protocols (see below). It has been known for more than 10 years that *Cyp7a1* mRNA is relatively unstable, putatively related to the circadian rhythm of its expression.[56] We therefore might have missed the LXR-induced peak expression measured by others. In any case, our data demonstrate that Abca1-dependent HDL-formation is dispensable for biliary routing of cholesterol both as free cholesterol and after conversion to bile salts.

Interestingly, LXR activation by T0901317 stimulated hepatobiliary cholesterol excretion without influencing biliary phospholipid excretion. The latter is in accordance with unaltered *Mdr2* expression upon LXR activation. Biliary bile salt secretion, which constitutes a major driving force for biliary cholesterol and phospholipid secretion,[57] was either not affected or even slightly reduced in treated animals. Consequently, LXR activation leads to cholesterol hypersecretion into bile, as illustrated by the 3 to 4-fold increase in biliary phospho-lipid:cholesterol ratio in all T0901317-treated groups of mice. This suggests LXR-mediated upregulation of a specific process/transporter responsible for cholesterol disposal into bile. It has been suggested that Abcg5/Abcg8, Abc half transporters defective in β-sitosterolemia,[44,45] may have a role in this process.[15,16] Direct evidence for this role, however, is still lacking. As recently reported[46] and confirmed in this study, expression of *Abcg5* and *Abcg8* is indeed induced in livers of LXR-treated mice. In a recent study we found a relationship between rates of biliary cholesterol excretion and hepatic *Abcg5/8* expression in different mouse models of biliary cholesterol hypersecretion.[58] However, there are also models of cholesterol hyper-secretion, most notably the diosgenin-fed mouse, in which *Abcg5/8* expression remains unaffected. Thus, direct proof for a role of these transporters in LXR induced cholesterol hypersecretion will have to await studies in *Abcg5/8* knockout mice.

It has previously been reported that, besides its effects on genes involved in cholesterol transport, LXR activation also affects fatty acid metabolism by both Srebp-1c dependent[42] and -independent mechanisms[30] and causes hepatic steatosis.[29] We did find dramatic increases in hepatic triglyceride contents upon T0901317-treatment in all groups, including the *Abca1*$^{-/-}$ mice, at least partially accounting for the increases in liver weight/body weight ratios. Overall gene expression patterns were also similar to results reported by other groups,[23,29,30,43] although generally less pronounced inductions upon T0901317-treatment were observed. However, for some genes (*e.g*. lipoprotein lipase, phospholipid transfer protein) we did find high levels of induction.[41] The modest increase in the other genes may be caused by a longer period of time between last dosage of the agonist and tissue sampling in our experiments, or by different application modes (gavage *vs*. diet). From the differences between C57BL/6J and DBA/1 wild-type mice and from quantitative differences between other studies,[23,29,30,43] it can furthermore be concluded that strain-specific factors may also be involved in reported differences in gene expression patterns upon LXR activation.

Because of its prominent position in controlling cholesterol homeostasis, pharmacological activation of LXR is currently widely discussed as a promising tool to raise HDL, improve RCT and therefore inhibit or prevent the development of atherosclerosis. In this study we demonstrated that LXR activation by T0901317 leads to similarly increased rates of hepato-biliary cholesterol output and increased fecal sterol loss in wild-type and *Abca1*$^{-/-}$ mice. The underlying LXR-dependent mechanism is thus independent from (Abca1-mediated) elevation of plasma HDL levels and the (putative) role of Abca1 in intestinal cholesterol absorption. In addition, LXR activation was associated with stimulation of net cholesterol loss via the intestine, indicating the presence of additional pathways for direct removal of cholesterol from the body.

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