DOI 10.1002/mnfr.201700055 *Mol. Nutr. Food Res.* *61*, *9*, 2017, 1700055

RESEARCH ARTICLE

Phytosterol-mediated inhibition of intestinal cholesterol absorption in mice is independent of liver X receptor

Lídia Cedó[1,2], David Santos[1,2], Iziar A. Ludwig[3], Reija Silvennoinen[4], Annabel García-León[1,5], Leena Kaipiainen[6], José M. Carbó[7], Annabel F. Valledor[7], Helena Gylling[6], Maria-José Motilva[3], Petri T. Kovanen[4], Miriam Lee-Rueckert[4], Francisco Blanco-Vaca[1,2,5] and Joan Carles Escolà-Gil[1,2,5]

[1] Institut d'Investigacions Biomèdiques (IIB) Sant Pau, Barcelona, Spain
[2] CIBER de Diabetes y Enfermedades Metabólicas Asociadas, CIBERDEM, Hospitalet de Llobregat, Spain
[3] Food Technology Department, UTPV-XaRTA, Agrotecnio Center, University of Lleida, Lleida, Spain
[4] Wihuri Research Institute, Helsinki, Finland
[5] Departament de Bioquímica, Biología Molecular i Biomedicina, Universitat Autònoma de Barcelona, Barcelona, Spain
[6] University of Helsinki and Helsinki University Central Hospital, Department of Internal Medicine, Helsinki, Finland
[7] Department of Cellular Biology, Physiology and Immunology, School of Biology, University of Barcelona, Barcelona, Spain

Scope: Previous studies have proposed that phytosterols activate liver X receptors (LXR) in the intestine, thereby reducing intestinal cholesterol absorption and promoting fecal cholesterol excretion.

Methods and results: In the present study, we examined the effects of dietary phytosterol supplementation on intestinal cholesterol absorption and fecal neutral sterol excretion in LXRαβ-deficient mice, and wild-type mice treated with synthetic high-affinity LXRαβ agonists. LXRαβ deficiency led to an induction of intestinal cholesterol absorption and liver cholesterol accumulation. Phytosterol feeding resulted in an approximately 40% reduction of intestinal cholesterol absorption both in wild-type and LXRαβ-deficient mice, reduced dietary cholesterol accumulation in liver and promoted the excretion of fecal cholesterol-derived compounds. Furthermore, phytosterols produced additive inhibitory effects on cholesterol absorption in mice treated with LXRαβ agonists.

Conclusions: Our data confirm the effect of LXR in regulating intestinal cholesterol absorption and demonstrate that the cholesterol-lowering effects of phytosterols occur in an LXR-independent manner.

Received: January 17, 2017
Revised: February 24, 2017
Accepted: February 27, 2017

Keywords:
Cholesterol / Intestine / Liver X receptor / Mice / Phytosterols

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Dietary consumption of phytosterols is a recommended therapeutic option to decrease LDL cholesterol [1, 2]. Based on the structural similarity between phytosterols and cholesterol, the competition between both sterols for incorporation into mixed micelles has been proposed as one of the main mechanisms underlying the hypocholesterolemic effect of plant sterols [3, 4]. However, phytosterols do not need to be present in the intestinal lumen simultaneously with cholesterol to inhibit its absorption, and therefore other potential mechanisms involving sterol transporters in the enterocyte have been proposed [3, 4].

The liver X receptors α and β (LXRαβ) are oxysterol-activated nuclear receptors that regulate the expression

Correspondence: Dr. Joan Carles Escolà-Gil or Dr. Francisco Blanco-Vaca
E-mail: jescola@santpau.cat or fblancova@santpau.cat

Abbreviations: **ABC**, ATP-binding cassette; **LXR**, liver X receptor; **NPC1L1**, Niemann-pick C1-like 1; **TICE**, transintestinal cholesterol excretion

of a number of genes involved in intestinal cholesterol absorption. Indeed, overexpression of intestinal LXRα down-regulated intestinal cholesterol absorption by increasing the expression of ATP-binding cassette (ABC) transporter A1 and the heterodimer ABCG5/G8 [5]. Consistent with these findings, LXRα-deficient mice also showed increased cholesterol absorption [6].

Several studies have reported that phytosterols such as sitosterol, sitostanol, stigmasterol, fucosterol, and other phytosterol metabolites act as LXR ligands, thereby inducing the expression of some critical LXR target genes, such as ABCA1 and ABCG5/G8 [7–10]. On the other hand, phytosterols have been shown to inhibit the generation of 27-hydroxycholesterol by sterol 27-hydroxylase, thereby reducing LXRα-mediated *ABCA1* expression and basolateral secretion of cholesterol in human Caco-2 enterocytes [11]. A recent report has shown that, under basal conditions, 35–40% of daily fecal neutral sterol excretion is derived from direct transintestinal cholesterol excretion (TICE) both in mice and humans [12]. LXRαβ activation also promotes TICE in mice [13]. Interestingly, phytosterols also promote TICE excretion that was, a least in part, dependent on ABCG5/G8 [14]. Beyond TICE, dietary phytosterols seem to reduce intestinal cholesterol absorption independently of ABCA1 and ABCG5/G8 [15–17]. Therefore, although significant evidence supports that phytosterols regulate LXR activity in vitro that may inhibit intestinal cholesterol absorption and promote fecal cholesterol excretion, this hypothesis has never been evaluated in vivo in genetically modified mice for LXRαβ.

In the present study, we investigated the effects of dietary phytosterols on fractional intestinal cholesterol absorption and fecal cholesterol-derived sterol excretion in LXRαβ-deficient mice, and we also evaluated the effects of the combined administration of phytosterols and a synthetic high-affinity LXRαβ agonist in wild-type mice.

2 Materials and methods

2.1 Mice and diet

C57BL/6J wild-type mice and apoE-deficient mice were obtained from Jackson Laboratories (Bar Harbor, ME; #000664 and #002052), and LXRαβ-deficient mice of the C57BL/6 background were kindly donated by Dr. David Mangelsdorf (UT Southwestern Medical Center, Dallas, TX). LXRα and LXRβ expression was determined in liver and small intestine by RT-PCR analyses and the absence of LXRs was confirmed in LXRαβ-deficient animals. All animal procedures were conducted in accordance with published regulations and reviewed and approved by the Institutional Animal Care Committee of the Institut de Recerca of the Hospital de la Santa Creu i Sant Pau (ref. 7281). Female mice were used because of their higher level of intestinal cholesterol absorption compared to males, thereby increasing the efficacy of phytosterols in reducing the intestinal cholesterol absorption [18]. The mice (4–6 months old) were kept in a temperature-controlled (22°C) room with a 12-hour (h) light/dark cycle. Food and water were provided ad libitum. Two different experiments were conducted. In the first, wild-type and LXRαβ-deficient mice were randomized into three groups and fed for 4 weeks with either: (i) a Western-type diet containing 21% fat and 0.2% cholesterol and less than 0.01% of phytosterols (TD.88137, Harlan Teklad, Madison, WI), which acted as the control; (ii) the Western-type diet supplemented with 2% phytosterols composed of 80% β-sitosterol, 11% β-sitostanol, and 7% campesterol (a detailed composition of the mixture, which considerably differs from the manufacturers specification, is shown in Supporting Information Table 1; Lipotec SAU, Gavà, Spain) [19]; or (iii) the Western-type diet supplemented with 0.005% ezetimibe (MSD-SPL, Hoddesdon, UK). Diets were prepared and mixed with phytosterols or ezetimibe by Mucedola SRL (Settimo Milanese, Milan, Italy). In the second experiment, wild-type mice were randomized into two groups and fed for 4 weeks with diets i and ii, as described above. The last 4 days of diet administration, mice were divided into two additional groups and administered an intragastric dose of 10 mg/kg of the synthetic high-affinity LXRαβ agonist T0901317 (Cayman Chemicals, Ann Arbor, MI) or vehicle (1% w/v carboxymethyl cellulose). Food intake and body weight were monitored in all experimental groups.

2.2 Fractional intestinal cholesterol absorption

The efficiency of intestinal cholesterol absorption was determined by the fecal dual isotope ratio method and using [^{3}H] sitostanol as a nonabsorbable recovery standard. The mice received a gastric bolus of 100 μL of olive oil containing 1 μCi of [4-^{14}C] cholesterol (Perkin Elmer, Waltham, MA) together with 2 μCi of [5,6-^{3}H] sitostanol (American Radiolabeled Chemicals, St. Louis, MO) [20]. The mice were then individually housed in metabolic cages, and their stools were collected over the next 2 days. The mice were then euthanized and exsanguinated with cardiac puncture and their livers and small intestines were removed. Fecal and liver lipids were extracted with isopropyl alcohol-hexane (2:3, v/v). The lipid layer was collected and evaporated, and [^{3}H] and [^{14}C] radioactivities were measured by liquid scintillation counting. The fractional cholesterol absorption was calculated according to the formula: [([^{14}C]/[^{3}H] dose ratio – [^{14}C]/[^{3}H] feces ratio)/[^{14}C]/[^{3}H] dose ratio] × 100. Hepatic [^{14}C] cholesterol was calculated as percentage of administered dose. There was a high correlation between direct [^{14}C]/[^{3}H] measurements of fecal lipid extracts and those determined after thin layer chromatography ($n = 10$; R square = 0.92; $p < 0.0001$).

2.3 Lipid analyses

Total serum cholesterol, HDL cholesterol, and triglycerides were determined enzymatically using commercial kits

adapted to a COBAS 6000 autoanalyzer (Roche Diagnostics, Rotkreuz, Switzerland). Triglyceride determinations were corrected for free glycerol. Hepatic lipids were extracted with isopropyl alcohol-hexane (2:3, v/v) [20]. The lipid layer was collected, evaporated, and resuspended in cholate 0.5% w/v, then cholesterol and triglycerides were determined using commercial kits adapted to the COBAS 6000 autoanalyzer.

Feces were collected for 48 h from the individually housed mice, dried for 24 h at 50°C, pulverized, and divided into two parts, one for the determination of bile acids and the other for the analysis of neutral sterols. Neutral sterols were extracted and analyzed by gas-liquid chromatography [21] in a system (Agilent 6890N Network GC System, Agilent Technologies, Santa Clara, CA) equipped with a 50 m nonpolar Ultra 2 capillary column. Standards (Sigma-Aldrich, St. Louis, MO; and Steraloids Ltd., Newport, RI) were run to identify the neutral sterols cholesterol and coprostanol, and the plant sterols campesterol, campestanol, stigmasterol, sitosterol, sitostanol, and avenasterol.

2.4 Bile acid analyses

Silylation of biliary acids was carried out according to Mosele et al. [22] with some modifications. Briefly, 200 μL of pyridine and 100 μL of N-methyl-N- (trimethylsilyl) trifluoroacetamide (both from Sigma-Aldrich) were added to vials containing 10 mg of feces, vortexed and then maintained during 30 min at 60°C. After silylation, samples were centrifuged 10 min at 8784 g at room temperature. The supernatants were analyzed by GC system (Agilent 6890N, Agilent Technologies) coupled to a mass spectrometer (Agilent 5973 MSD, Agilent Technologies). Samples were injected into a capillary column HP-5MS (30 m × 0.25 mm × 0.25 id) and the analysis of biliary acids was performed as follows. Helium was the carrier gas (1 mL/min). Injection was carried out with a split injector (1:10) at 250°C. The column temperature started at 240°C and was raised at a rate of 20°C/min until it reached 290°C and maintained for 2 min, raised to 295°C at a rate of 1°C/min, and then raised at a rate of 25°C/min to 310°C, and maintained for 10 min (total run time 20.1 min). Peak identification was based on comparison of retention times and mass fragmentation patterns with reference compounds. All quantifications were performed in selected ion monitoring mode using calibration curves generated from different known concentrations of commercial standards. For fecal biliary acids quantification, commercial standards of cholic acid, deoxycholic acid, chenodeoxycholic acid, lithocholic acid, and ursodeoxycholic acid (Sigma-Aldrich) were used.

2.5 Quantitative real-time PCR analyses

Total RNA from liver and small intestine was extracted using TRIzol LS Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions, and purified using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). cDNA was generated using Oligo$(dT)_{23}$ and dNTPs mix (Sigma-Aldrich) and M-MLV Reverse Transcriptase RNase H Minus Point Mutant (Promega, Madison, WI), and was subjected to quantitative real-time PCR amplification using the TaqMan Master Mix (Applied Biosystems, Foster City, CA) and specific TaqMan probes (Applied Biosystems) for *abca1* (Mm004426464_m1), *abcg5* (Mm00446241_m1), *abcg8* (Mm00445970_m1), *acaca* (Mm01304257_m1), *cyp7a1* (Mm00470430_m1), *fasn* (Mm01253292_m1), *npc1l1* (Mm01191972_m1), *nr1h3* (Mm00443451_m1), and *gapdh* (Mm99999915_g1) as the internal control gene. Reactions were run on a CFX96TM Real-Time System (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. cDNA was also subjected to quantitative real-time PCR amplification using the SYBR green master mix (Applied Biosystems) and specifically designed primers for *nr1h2* (Fw: CATTGCGACTCCAGGACAAGA and Rev: CCCAGATCTCGGACAGCAAG) and *l14* as the internal control gene (Fw:TCCCAGGCTGTTAACGCGGT and Rev: GCGCTGGCTGAATGCTCTG). Reactions were run on an ABI PRISM 7900 HT (Applied Biosystems) according to the manufacturer's instructions. The relative mRNA expression levels were calculated using the ΔΔCt method [23].

2.6 Statistical methods

The sample size of five individuals allowed at least 95% power to detect a statistically significant difference among groups of 10% in intestinal cholesterol absorption, and a standard deviation of 4%, assuming a Type I error of 0.05 (two-sided). Two-way ANOVA was used for comparing differences among groups with the factors of genotype, treatment, and interaction. Tukey's post-test was used for comparing differences among treatments in both wild-type and LXRαβ-deficient mice; and Sidak's post-test was used for comparing wild-type versus LXRαβ-deficient mouse data. One-way ANOVA with Tukey's post-test was used to compare differences among vehicle, LXR agonist, vehicle + phytosterols and LXR agonist + phytosterols groups. GraphPad Prism 4.0 software (GraphPad, San Diego, CA) was used to perform all statistical analyses. A p value of <0.05 was considered statistically significant.

3 Results

3.1 Phytosterol-mediated inhibition of intestinal cholesterol absorption does not require functional LXRαβ

First, we measured the effect of phytosterol supplementation on the intestinal cholesterol absorption in wild-type and apoE-deficient mice fed with a Western diet as low- and high-absorbers, respectively [18]. As expected, the absence

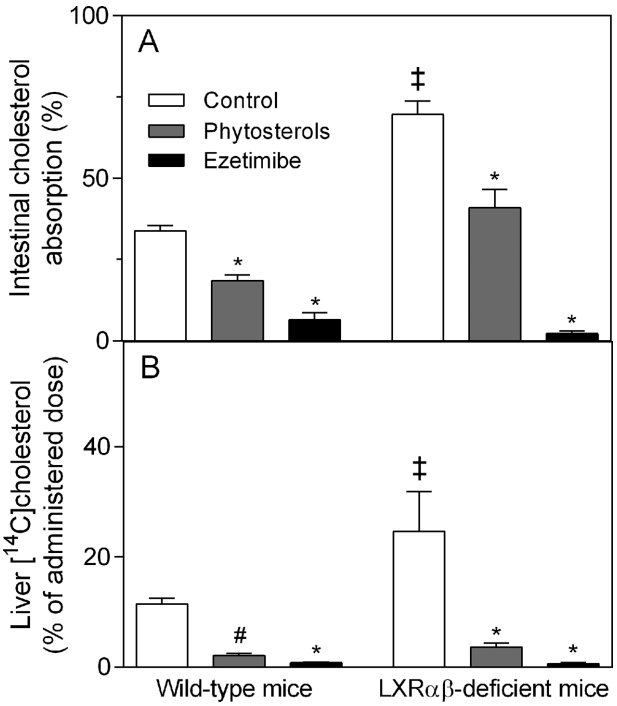

Figure 1. (A) Effects of phytosterols on the fractional intestinal cholesterol absorption in wild-type and LXRαβ-deficient mice treated with a Western-type diet (control) or a Western diet supplemented with either 2% phytosterols or 0.005% ezetimibe. (B) Intestine-derived [^{14}C] cholesterol radioactivity in the liver at 48 h. Values are the mean ± SEM of six mice/group. Two-way ANOVA and Tukey's and Sidak's multiple comparisons test were used to compare differences among groups in both wild-type and LXRαβ-deficient mice. $^{*}p < 0.05$ and $^{\#}p < 0.1$, respectively, versus control; $^{‡}p < 0.05$ between LXRαβ-deficient mice and control wild-type mice. Since ezetimibe was used as a positive control for intestinal cholesterol absorption, we do not show the comparison between ezetimibe and phytosterols groups.

of apoE increased by twofold the rate of cholesterol absorption. Importantly, cholesterol absorption was reduced by 40% in either group of phytosterol-treated mice regardless of their basal cholesterol absorption levels and a full inhibitory effect was already achieved when mice were given 1% of dietary phytosterols (Supporting Information Fig. 1A). Then, two independent groups of wild-type and LXRαβ-deficient mice were treated for 4 weeks with the phytosterol-containing or the nonsupplemented (control) Western diet to ascertain whether phytosterols affect intestinal cholesterol absorption in the setting of LXRαβ deficiency. The fecal dual isotope method was applied to measure the fractional cholesterol absorption over 48 h. As shown in Fig. 1A, the percentage of cholesterol absorbed was significantly higher in the control LXRαβ-deficient mice compared to that of wild-type mice. Notably, addition of phytosterols to the control diet reduced intestinal cholesterol absorption by 40% in both genotypes. The amount of fecal [^{3}H] sitostanol was not affected by phytosterol supplementation (91.1 ± 4.7 and 89.3 ± 2.3% of administered dose in phytosterol-treated versus control wild-type mice, and 80.0 ± 6.9 and 85.1 ± 10.7% of administered dose in phytosterols-treated versus control LXRαβ-deficient mice). Furthermore, serum [^{3}H] sitostanol levels were very low (less than 0.3% of administered dose per mL of serum in all groups) and were not affected by phytosterol supplementation. Therefore, sitostanol was only poorly recovered from the systemic circulation and had a transport rate through the gastrointestinal tract that was independent of the treatment.

Since LXR regulation affects key target genes involved in the intestinal cholesterol homeostasis such as *abca1*, *abcg5*, and *abcg8* [24], we aimed to evaluate whether the phytosterol supplementation could upregulate the expression of these genes in this relevant tissue. As previously reported [25], the expression of *abca1* was regulated in a tissue-specific manner in LXRαβ-deficiency. Therefore, intestinal *abca1* was upregulated in LXRαβ-deficient mice, whereas hepatic *abca1* was downregulated (Table 1). However, the supplementation with phytosterols did not significantly affect the expression of these genes in either group (Table 1).

Two additional groups of wild-type and LXRαβ-deficient mice were treated with ezetimibe and used as a positive control for inhibiting intestinal cholesterol absorption without affecting LXR activity, since the drug blocks the internalization of the NPC1L1 (Niemann-pick C1-like 1)/cholesterol complex and severely impairs intestinal cholesterol absorption independently of its baseline levels [12,26]. As expected, ezetimibe blocked almost completely intestinal cholesterol absorption in both genotypes (Fig. 1A).

3.2 Dietary phytosterols impair cholesterol accumulation in liver from wild-type and LXRαβ-deficient mice

The mice tolerated well the different diets, and no significant differences were observed between body weight and food intake in the different groups (Table 2). Consistent with our previous reports on wild-type mice [18], no changes in serum lipids were observed between phytosterol-treated and untreated mice (Table 2). This has been previously described in animals with a nonexpanded whole-body pool of cholesterol [18, 27–30], as occurred in our wild-type mice (Table 2). LXRαβ-deficient mice exhibited a moderate, but significant, increase in non-HDL-cholesterol compared with their wild-type counterparts, but phytosterol treatment did not significantly affect this parameter (Table 2).

We also examined the potential effect of phytosterols in the hepatic cholesterol accumulation. For this purpose, radioactivity was measured in the liver 48 h after the intragastric administration of radiolabeled cholesterol. Hepatic [^{14}C] cholesterol levels were higher in the LXRαβ-deficient mice compared to the wild-type mice, whereas they decreased in the phytosterol-treated mice independently of the absence of LXRαβ (Fig. 1B). The LXRαβ-deficient mice also showed a higher level of total hepatic cholesterol concomitant

Table 1. Relative intestinal and liver mRNA levels in wild-type and LXRαβ-deficient mice treated with phytosterols or ezetimibe

	Wild-type mice			LXRαβ-deficient mice		
	Control	Phytosterols	Ezetimibe	Control	Phytosterols	Ezetimibe
INTESTINE						
abca1	1.00 ± 0.06	0.96 ± 0.09	0.83 ± 0.10	2.18 ± 0.34‡	2.43 ± 0.22	1.45 ± 0.22*
abcg5	1.00 ± 0.22	0.81 ± 0.09	0.84 ± 0.12	1.12 ± 0.16	1.36 ± 0.07	0.66 ± 0.03
abcg8	1.00 ± 0.19	0.82 ± 0.13	0.72 ± 0.08	1.19 ± 0.26	1.28 ± 0.16	0.62 ± 0.06
npc1l1	1.00 ± 0.19	1.62 ± 0.08	2.32 ± 0.36*	1.35 ± 0.38	2.39 ± 0.43	0.82 ± 0.19
LIVER						
abca1	1.00 ± 0.02	0.97 ± 0.13	0.98 ± 0.06	0.55 ± 0.04‡	0.69 ± 0.03	0.94 ± 0.15*
abcg5	1.00 ± 0.11	0.98 ± 0.06	1.09 ± 0.13	0.37 ± 0.03	1.00 ± 0.17	1.79 ± 0.78*
abcg8	1.00 ± 0.03	1.15 ± 0.19	1.31 ± 0.25	0.39 ± 0.01	1.12 ± 0.17	2.31 ± 0.99*
cyp7a1	1.00 ± 0.24	0.54 ± 0.08	0.90 ± 0.25	1.09 ± 0.37	1.20 ± 0.04	1.17 ± 0.42

Values are mean ± SEM of five mice/group. Two-way ANOVA and Tukey's and Sidak's multiple comparisons test were used to compare differences among groups in both wild-type and LXRαβ-deficient mice. Differences among phytosterols, ezetimibe, and control diets are shown in each mouse genotype. Since ezetimibe was used as a positive control for intestinal cholesterol absorption, we do not show the comparison between ezetimibe and phytosterols groups. *$p < 0.05$ versus control; ‡$p < 0.05$ between LXRαβ-deficient mice and control wild-type mice.

with reduced hepatic triglyceride levels and with decreased expression of the main hepatic genes involved in triglyceride synthesis (Table 2 and Supporting information Table 2). Furthermore, the liver weight and the hepatic content of triglycerides in the phytosterol-treated mice did not differ significantly from those of the control mice (Table 2). Hepatic cholesterol content was lower in the phytosterol-treated mice compared to the control mice, although this change was only significant in the LXRαβ-deficient mice (Table 2).

As expected, ezetimibe caused significant reductions in hepatic [^{14}C] cholesterol uptake and cholesterol levels from both genotypes (Fig. 1B and Table 2). Hepatic *abcg5/g8* was upregulated by ezetimibe in the LXRαβ-deficient mice (Table 1), which could contribute to enhance biliary cholesterol excretion and, consequently, its fecal excretion (Table 3). This change has been previously reported in mice in which *abcg5/g8* is not highly expressed [31], as occur in LXRαβ-deficient mice.

3.3 Phytosterol-mediated increase of fecal cholesterol excretion is independent of LXRαβ

We next investigated the effects of phytosterols on the fecal excretion of cholesterol and bile acids in LXRαβ-deficient and wild-type mice (Table 3). In line with the increased fecal excretion of cholesterol reported in LXR agonist-treated wild-type mice [32, 33], gas–liquid chromatography analyses of feces excreted over 48 h showed reduction in fecal cholesterol and cholesterol-derived neutral sterols in the LXRαβ-deficient mice compared to the wild-type mice (Table 3).

Table 2. Serum and liver lipids in wild-type and LXRαβ-deficient mice treated with phytosterols or ezetimibe

	Wild-type mice			LXRαβ-deficient mice		
	Control	Phytosterols	Ezetimibe	Control	Phytosterols	Ezetimibe
Food intake (g/day)	2.77 ± 0.25	2.75 ± 0.11	3.39 ± 0.18	3.16 ± 0.37	3.65 ± 0.32	3.39 ± 0.35
Body weight (g)	25.48 ± 1.47	22.31 ± 1.45	24.00 ± 0.67	24.47 ± 1.76	25.58 ± 0.70	22.60 ± 1.50
Serum cholesterol (mM)	3.11 ± 0.19	2.67 ± 0.14	2.75 ± 0.22	3.73 ± 0.13‡	3.50 ± 0.12	3.19 ± 0.37
HDL cholesterol (mM)	2.85 ± 0.12	2.38 ± 0.12	2.49 ± 0.23	2.66 ± 0.17	2.96 ± 0.09	2.43 ± 0.70
Non-HDL cholesterol (mM)	0.26 ± 0.08	0.28 ± 0.06	0.26 ± 0.05	0.95 ± 0.19‡	0.55 ± 0.06	0.76 ± 0.36
Serum triglycerides (mM)	0.71 ± 0.03	0.74 ± 0.04	0.79 ± 0.07	0.54 ± 0.04	0.49 ± 0.04	0.69 ± 0.09
Liver weight (g)	1.28 ± 0.07	1.07 ± 0.03	1.04 ± 0.07	1.57 ± 0.09‡	1.52 ± 0.04	1.31 ± 0.11
Liver cholesterol (μmol/g)	4.46 ± 0.43	2.97 ± 0.20	2.45 ± 0.14	8.26 ± 1.67‡	3.90 ± 0.28*	2.39 ± 0.55*
Liver triglycerides (μmol/g)	11.72 ± 0.79	9.03 ± 2.55	4.12 ± 0.94*	5.91 ± 1.01‡	6.21 ± 0.19	5.75 ± 2.48

Values are mean ± SEM of six mice/group. Two-way ANOVA and Tukey's and Sidak's multiple comparisons test were used to compare differences among groups in both wild-type and LXRαβ-deficient mice. Differences among phytosterols, ezetimibe, and control diets are shown in each mouse genotype. Since ezetimibe was used as a positive control for intestinal cholesterol absorption, we do not show the comparison between ezetimibe and phytosterols groups. *$p < 0.05$ versus control; ‡$p < 0.05$ between LXRαβ-deficient mice and control wild-type mice.

Table 3. Fecal neutral sterols and bile acids in wild-type and LXRαβ-deficient mice treated with phytosterols or ezetimibe

Sterols (mg/g feces)	Wild-type mice			LXRαβ-deficient mice		
	Control	Phytosterols	Ezetimibe	Control	Phytosterols	Ezetimibe
Cholesterol	13.89 ± 0.69	21.60 ± 0.42*	27.98 ± 1.01*	6.76 ± 0.50‡	20.48 ± 0.99*	43.71 ± 2.22*
Coprostanol	0.025 ± 0.014	0.157 ± 0.021	0.064 ± 0.024	0.008 ± 0.003	1.238 ± 1.007	0.303 ± 0.255
Total cholesterol-derived neutral sterols	13.92 ± 0.71	21.76 ± 0.41*	28.04 ± 1.03*	6.76 ± 0.50‡	21.72 ± 1.15*	44.01 ± 1.97*
Campesterol	0.06 ± 0.01	12.10 ± 0.11*	0.07 ± 0.01	0.07 ± 0.01	10.93 ± 0.68*	0.13 ± 0.01
Campestanol	0.008 ± 0.001	2.08 ± 0.02*	0.010 ± 0.001	0.009 ± 0.001	1.93 ± 0.05*	0.018 ± 0.004
Stigmasterol	0.062 ± 0.004	1.102 ± 0.006*	0.098 ± 0.008	0.005 ± 0.001	0.96 ± 0.06*	0.025 ± 0.005
Sitosterol	0.05 ± 0.01	137.20 ± 1.26*	0.14 ± 0.02	0.07 ± 0.01	124.50 ± 7.52*	0.17 ± 0.02
Sitostanol	0.03 ± 0.01	18.93 ± 0.17*	0.04 ± 0.01	0.03 ± 0.01	17.60 ± 0.70*	0.06 ± 0.01
Avenasterol	0.03 ± 0.01	1.23 ± 0.02*	0.06 ± 0.01	0.03 ± 0.01	1.14 ± 0.07*	0.11 ± 0.01
Total plant phytosterols	0.23 ± 0.01	172.60 ± 1.60*	0.42 ± 0.03	0.22 ± 0.02	157.10 ± 9.06*	0.52 ± 0.05
Bile acids (μmol/g feces)						
Lithocholic acid	0.67 ± 0.03	0.61 ± 0.11	0.52 ± 0.00	0.80 ± 0.09	0.96 ± 0.17	0.51 ± 0.01
Deoxycholic acid	2.12 ± 0.22	1.08 ± 0.07*	1.56 ± 0.23	4.35 ± 0.41‡	1.77 ± 0.28*	1.26 ± 0.19*
Chenodeoxycholic acid	0.96 ± 0.16	0.53 ± 0.01	0.94 ± 0.09	2.98 ± 0.55‡	0.86 ± 0.01*	0.93 ± 0.09*
Cholic acid	0.77 ± 0.18	0.53 ± 0.03	0.77 ± 0.11	2.41 ± 0.78‡	0.87 ± 0.12*	0.81 ± 0.09*

The cholesterol-derived neutral sterols included cholesterol and coprostanol, and the plant-derived phytosterols included campesterol, campestanol, stigmasterol, sitosterol, sitostanol, and avenasterol. Ursodeoxycholic acid was not quantified as it was below the detection limit. Values are the mean ± SEM of four mice/group. Two-way ANOVA and Tukey's and Sidak's multiple comparisons test were used to compare differences among groups in both wild-type and LXRαβ-deficient mice. Since ezetimibe was used as a positive control for intestinal cholesterol absorption, we do not show the comparison between ezetimibe and phytosterols groups. *$p < 0.05$ versus control; ‡$p < 0.05$ between LXRαβ-deficient mice and control wild-type mice.

Importantly, the addition of phytosterols to the control diet increased the fecal excretion of cholesterol-derived compounds both in LXRαβ-deficient and wild-type mice (Table 3). Furthermore, LXRαβ-deficient mice showed an increased fecal excretion of bile acids, whereas the phytosterol-treated mice exhibited reduced fecal excretion of bile acids both in LXRαβ-deficient and wild-type mice, mainly due to a decrease in deoxycholic acid (Table 3).

3.4 Treatment with phytosterols exerts additive effects on intestinal cholesterol absorption in mice treated with synthetic high-affinity LXRαβ agonist

The LXRαβ agonist T0901317 inhibited cholesterol absorption by 35% in wild-type mice fed the Western diet and a full inhibitory effect was achieved with a dose of 10 mg/kg/day (Supporting Information Fig. 1B), which was used to minimize the side effects of LXR activation on liver triglyceride accumulation [24]. We then investigated the potential effects of phytosterols on intestinal cholesterol absorption in LXR-ligand-treated animals. For this purpose, wild-type mice fed the control or the phytosterol-containing diets were treated with either the high-affinity LXR agonist T0901317 or vehicle. As expected, the LXR agonist increased triglyceride levels in wild-type mice without affecting body weight or food intake (Table 4) [20, 34]. However, hepatic cholesterol content decreased by 60% (Table 4), presumably due to reduced intestinal cholesterol absorption (Fig. 2A and [32, 33]) and downregulation of hepatic cholesterol synthesis concomitant with an increased bile acid synthesis [33, 35]. More importantly, phytosterols produced an additive effect on the T0901317-dependent inhibition of cholesterol absorption (Fig. 2A). Furthermore, phytosterol treatment enhanced fecal excretion of cholesterol-derived neutral sterols in LXR agonist-treated animals, although this change was not significant when compared with mice given phytosterols alone (Table 5). Notably, whereas T0901317 upregulated the LXR target gene *abca1* in the small intestine and *abcg5* and *abcg8* in the liver, treatment with phytosterols did not exert any further effect on the LXR-mediated changes (Table 6). Similarly, T0901317 upregulated hepatic *acaca*, but phytosterols did not exert any further effect at this level (Supporting Information Table 3).

4 Discussion

Strong evidence in vitro supports the hypothesis that phytosterols act as ligands for LXRs [7–11], thereby suggesting that they may regulate intestinal cholesterol absorption in vivo through an LXRαβ-sensitive pathway. However, this hypothesis has never been evaluated in genetically modified mice for LXRαβ. To the best of our knowledge, the present study demonstrates for the first time that disruption of LXRαβ expression did not affect the ability of phytosterols to inhibit

Table 4. Serum and liver lipids in wild-type mice treated with the vehicle, LXRαβ agonist T0901317, phytosterols, or both

	Vehicle	LXR agonist	Vehicle + phytosterols	LXR agonist + phytosterols
Food intake (g/day)	2.32 ± 0.12	2.10 ± 0.11	2.88 ± 0.30	2.32 ± 0.32
Body weight (g)	21.08 ± 0.15	23.18 ± 1.08	23.20 ± 0.48	23.46 ± 1.37
Serum cholesterol (mM)	3.87 ± 0.12	4.52 ± 0.33	3.39 ± 0.29	3.94 ± 0.34
HDL cholesterol (mM)	2.82 ± 0.15	3.28 ± 0.30	2.60 ± 0.25	2.66 ± 0.28
Non-HDL cholesterol (mM)	1.05 ± 0.18	1.24 ± 0.23	0.79 ± 0.07	1.28 ± 0.14
Serum triglycerides (mM)	0.56 ± 0.05	1.08 ± 0.11*	0.72 ± 0.22	0.85 ± 0.09
Liver weight (g)	1.05 ± 0.03	1.58 ± 0.01*	1.24 ± 0.08	1.52 ± 0.05*,‡
Liver cholesterol (μmol/g)	4.54 ± 0.18	2.35 ± 0.15*	2.67 ± 0.33*	2.05 ± 0.11*
Liver triglycerides (μmol/g)	12.92 ± 1.62	7.94 ± 1.23	10.76 ± 2.33	10.36 ± 0.80

Values are the mean ± SEM of five mice/group. ANOVA was used to compare differences among groups. *$p < 0.05$ versus vehicle; ‡$p < 0.05$ versus vehicle + phytosterols group.

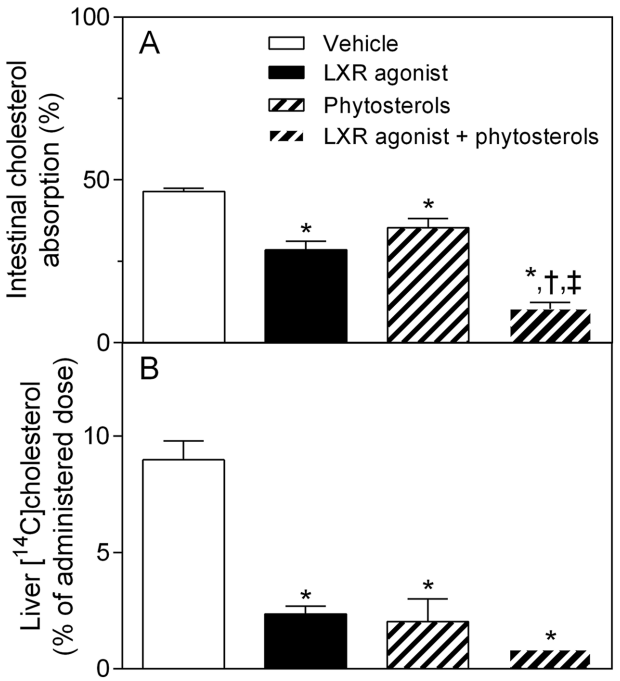

Figure 2. (A) Fractional intestinal cholesterol absorption in wild-type mice fed the Western-type diet or the Western diet supplemented with 2% phytosterols and treated with either LXR agonist T0901317 (10 mg/kg/day) or vehicle. (B) Intestinal-derived [^{14}C] cholesterol radioactivity levels in the mice livers at 48 h. Values are the mean ± SEM of five mice/group. ANOVA was used to compare differences among treatments. *$p < 0.05$ versus vehicle; †$p < 0.05$ versus LXR-treated group; ‡$p < 0.05$ versus vehicle + phytosterols group.

intestinal cholesterol absorption and accordingly to increase the fecal excretion of cholesterol-derived compounds in vivo. The phytosterols used in this study (mainly β-sitosterol, β-sitostanol, and campesterol) comprise more than 85% of the total phytosterols present in vegetable extracts [1]. These plant sterols were previously shown they could influence critical steps in cellular cholesterol homeostasis and have antiatherogenic and immunomodulatory effects in mice at the dose (2%) used in the present work [3]. In agreement with previous data [33], we also showed that cholesterol absorption was strongly increased in LXRαβ-deficient mice. More importantly, whereas our data confirm the effect of LXRαβ in regulating cholesterol homeostasis, the main finding of the present study is that phytosterols regulate intestinal cholesterol absorption via LXR-independent routes (Fig. 3). It should be noted that phytosterols have been reported to induce fecal cholesterol excretion via a nonbiliary TICE route [14]. Similarly, LXRαβ, ABCG5/G8, and NPC1L1 have been shown to play a role in regulating the TICE rate [12, 13]. Of note, although previous data clearly demonstrated a role for ABCG5/G8 in phytosterol-mediated TICE excretion, the changes in *abcg5/g8* expression were not associated with the increase in cholesterol flux through the pathway [14], which is consistent with our data. Although our experiments were not designed to assess the importance of such nonbiliary route, our data also suggest that one potential LXRαβ-independent mechanisms involved in the inhibitory effect of phytosterols on the fecal cholesterol excretion is the TICE route (Fig. 3). Furthermore, we also showed that administration of phytosterols to mice treated with an LXRαβ agonist resulted in further reduction of intestinal cholesterol absorption without enhancing the LXRαβ target gene expression, thereby confirming that the mechanisms whereby phytosterols affect cholesterol absorption are independent of LXRαβ. It should be noted that the combined treatment with LXRαβ agonist and phytosterols enhanced fecal cholesterol-derived neutral sterol excretion, but without producing additive effects. Since LXRαβ agonists may also negatively regulate hepatic cholesterol biosynthesis and induce bile acid synthesis [33, 35], the effects of phytosterols on fecal cholesterol excretion could be restricted under conditions of LXR activation.

It should be noted that the upregulation of *abcg5/g8* has been previously shown in mice given ezetimibe in which *abcg5/g8* is not highly expressed, as occur in our LXRαβ-deficient [31]. Furthermore, this upregulation was closely associated with the severe depletion of hepatic cholesterol in LXRαβ-deficient treated with ezetimibe. Of note,

Table 5. Fecal neutral sterols and bile acids in wild-type mice treated with LXRαβ agonist T0901317, phytosterols, or both

Sterols (mg/g feces)	Vehicle	LXR agonist	Vehicle + phytosterols	LXR agonist + phytosterols
Cholesterol	13.40 ± 0.87	16.80 ± 2.17	20.98 ± 0.47*	23.46 ± 0.45*,†
Coprostanol	0.05 ± 0.03	1.77 ± 1.70	1.73 ± 0.69	0.68 ± 0.22
Total cholesterol-derived neutral sterols	13.44 ± 0.86	18.57 ± 1.04*	22.71 ± 0.26*,†	24.15 ± 0.57*,†
Campesterol	0.06 ± 0.00	0.08 ± 0.01	12.10 ± 0.56*,†	11.59 ± 0.18*,†
Campestanol	0.02 ± 0.00	0.01 ± 0.00	2.17 ± 0.06*,†	2.00 ± 0.02*,†,‡
Stigmasterol	0.01 ± 0.00	0.01 ± 0.00	1.08 ± 0.04*,†	1.01 ± 0.02*,†
Sitosterol	0.09 ± 0.01	0.11 ± 0.01	135.3 ± 6.16*,†	130.6 ± 1.97*,†
Sitostanol	0.04 ± 0.00	0.04 ± 0.00	19.17 ± 0.71*,†	18.26 ± 0.23*,†
Avenasterol	0.03 ± 0.01	0.06 ± 0.01	1.24 ± 0.05*,†	1.07 ± 0.01*,†,‡
Total plant phytosterols	0.24 ± 0.02	0.32 ± 0.03	171.0 ± 7.54*,†	164.6 ± 2.42*,†
Bile acids (μmol/g feces)				
Lithocholic acid	0.67 ± 0.02	0.83 ± 0.05*	0.63 ± 0.03†	0.57 ± 0.03†
Deoxycholic acid	2.01 ± 0.07	2.55 ± 0.33	1.17 ± 0.03*,†	1.22 ± 0.12*,†
Chenodeoxycholic acid	0.79 ± 0.05	0.78 ± 0.06	0.68 ± 0.05	0.62 ± 0.02
Cholic acid	0.70 ± 0.05	0.65 ± 0.11	0.61 ± 0.01	0.52 ± 0.02

The cholesterol-derived neutral sterols included cholesterol and coprostanol, and the plant-derived phytosterols included campesterol, campestanol, stigmasterol, sitosterol, sitostanol, and avenasterol. Ursodeoxycholic acid was not quantified as it was below the detection limit. Values are the mean ± SEM of four mice/group. ANOVA was used to compare differences among groups. $^{*}p < 0.05$ versus vehicle; $^{\dagger}p < 0.05$ versus LXRαβ agonist-treated group; $^{\ddagger}p < 0.05$ versus vehicle + phytosterols group.

phytosterols also tended to increase hepatic *abcg5/g8* expression, but this change was not significant, thereby indicating that there is a common mechanism whereby hepatic cholesterol depletion increases the expression of *abcg5/g8*. The mechanisms by which ezetimibe upregulates hepatic *abcg5/g8* requires further investigation.

The intestinal-specific expression of LXRα has been shown to be inversely correlated with intestinal cholesterol absorption [5, 6], suggesting that our findings in LXRαβ-deficient mice are primarily due to the intestinal LXRα deficiency. In contrast to the beneficial effects mediated by LXR on cholesterol homeostasis, whole-body LXRαβ activation is usually associated with liver-specific side effects such as hepatic steatosis [34, 36], whereas LXRαβ deficiency downregulates genes responsible for fatty acid biosynthesis and reduces liver triglyceride accumulation [25, 37]. The absence of changes in the hepatic levels of triglycerides in phytosterol-treated wild-type mice provides additional evidence that phytosterols do not act as LXR activators in the liver.

Unlike in humans, phytosterols have a limited effect on the plasma levels of total cholesterol in mice regardless of reducing intestinal cholesterol absorption [18, 27–30]. Since cholesterol is predominantly associated with HDL in mice, it is plausible that with a nonexpanded whole-body pool of cholesterol and its very high turnover, as occurs in mice with very low non-HDL-cholesterol, the changes in net intestinal absorption may be translated into changes in cholesterol

Table 6. Relative intestinal and liver mRNA levels in wild-type mice treated with the vehicle, LXRαβ agonist T0901317, phytosterols, or both

	Vehicle	LXR agonist	Vehicle + phytosterols	LXR agonist + phytosterols
INTESTINE				
abca1	1.00 ± 0.09	2.89 ± 0.32*	0.61 ± 0.12†	3.24 ± 0.26*,‡
abcg5	1.00 ± 0.14	1.35 ± 0.14	0.69 ± 0.05†	1.23 ± 0.03
abcg8	1.00 ± 0.07	1.52 ± 0.11	0.80 ± 0.07†	1.93 ± 0.27*,‡
npc1l1	1.00 ± 0.24	0.76 ± 0.05	0.91 ± 0.08	1.14 ± 0.16
LIVER				
abca1	1.00 ± 0.15	1.49 ± 0.32	0.81 ± 0.06	1.15 ± 0.12
abcg5	1.00 ± 0.17	2.03 ± 0.09*	0.66 ± 0.03†	1.62 ± 0.04*,†,‡
abcg8	1.00 ± 0.18	1.77 ± 0.16*	0.74 ± 0.10†	1.64 ± 0.09*,‡
cyp7a1	1.00 ± 0.08	1.62 ± 0.28	0.99 ± 0.28	0.96 ± 0.26

Values are the mean ± SEM of five mice/group. ANOVA was used to compare differences among groups. $^{*}p < 0.05$ versus vehicle; $^{\dagger}p < 0.05$ versus LXR agonist-treated group; $^{\ddagger}p < 0.05$ versus vehicle + phytosterols group.

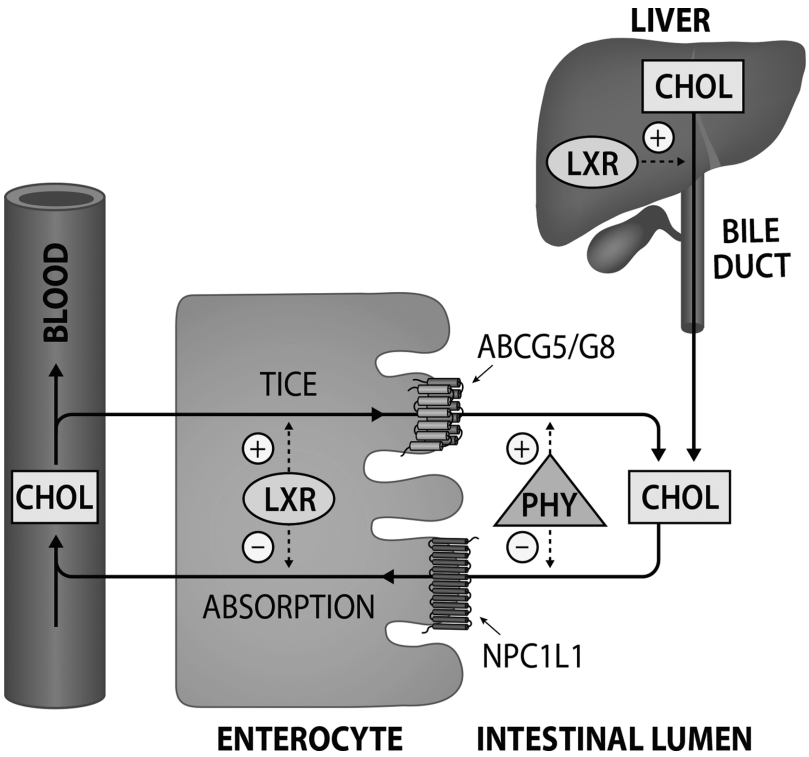

Figure 3. Phytosterols (PHY) and activation of intestinal liver X receptors (LXR) induce similar effects on cholesterol homeostasis in the intestine, which involves the flux of sterols between the enterocyte and the intestinal lumen regulated by the transporters NPC1L1 and ABCG5/G8. Activation of LXR in the enterocyte reduces intestinal cholesterol absorption and promotes transintestinal cholesterol excretion (TICE). In addition, activation of hepatic LXR stimulates biliary cholesterol secretion. By competing with intestinal cholesterol for incorporation into mixed micelles, dietary phytosterols induce a profound inhibitory effect on cholesterol absorption, such action taking place mainly in the intestinal lumen. Also, phytosterols have been reported to activate TICE but the exact mechanism of action remains unknown. The present study confirms the crucial role of LXR in the regulation of the whole-body cholesterol homeostasis. Notably, the results demonstrate that the cholesterol-lowering effects of phytosterols in vivo are regulated in an LXR-independent manner. Thus, dietary supplementation with phytosterols is sufficient to impair intestinal cholesterol absorption even when LXR activity is compromised.

levels in liver [28, 29], but not in serum [18, 27–30]. Furthermore, phytosterols did not affect serum and liver levels of triglycerides. The latter results contrasted with two previous reports in mice that showed lower plasma triglyceride levels after administration of phytosterols [28, 29], although liver triglycerides remained unchanged in one report [29]. Differences in the period of treatment and dietary fat composition could explain differences observed in the triglyceride homeostasis between our study and the two above-cited studies.

Beyond their effects on intestinal cholesterol absorption, phytosterols were able to reduce the fecal excretion of bile acids. Although these results could be considered somewhat unexpected, we and others have found similar changes in mice fed a phytosterol-containing diet [16, 30] and in mice treated with disodium ascorbyl phytostanol phosphate [38], as also shown in the present work. Of note, one report found that LXRα-deficient mice fed a cholesterol-containing diet displayed a more hydrophobic bile acid profile, which may promote intestinal cholesterol absorption [6]. It was recently shown in a mouse model of parenteral nutrition-associated liver disease that stigmasterol suppressed the expression of bile acid transporters and of LXR target genes in mice, resulting in cholestatic effects [39]. However, dietary stigmasterol is not transported to the liver in wild-type mice [18, 24]. We also found a significant increase in fecal bile acid excretion of the LXRαβ-deficient mice, mainly in the hydrophobic deoxycholic and chenodeoxycholic acids. Furthermore, phytosterols reduced the excretion of these fecal bile acids both in wild-type and LXRαβ-deficient mice, which was strongly correlated with cholesterol depletion in the liver, thereby suggesting that phytosterols, by reducing the accumulation of cholesterol in the liver, compromised the synthesis and excretion of bile acids [30].

In conclusion, while our study confirms that LXRαβ is crucial for the whole-body cholesterol homeostasis, it demonstrates that these important regulators of sterol transport in the intestine are not required for the phytosterol-mediated effects in reducing the intestinal absorption of cholesterol and so increasing its fecal excretion, at least in mice.

This work was partly funded by the Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, and FEDER "Una manera de hacer Europa," grants FIS 14–01648 (to F.B.-V.) and FIS 16–00139 (to J.C.E.-G.), grant FJCI-2014-20689 from the Ministerio de Economía, Industria y Competitividad (to I.A.L.) and grant 12/C/2015 from La Fundació la Marato TV3 (to F.B-V). CIBER de Diabetes y Enfermedades Metabólicas Asociadas is an Instituto de Salud Carlos III Project.

L.C., F.B.-V., and J.C.E.-G. conceived and designed the study, performed statistical analysis and drafted the manuscript. P.T.K., M.L.-R., H.G., and A.F.-V. participated in the design of the study and the edition of the manuscript. L.C., D.S., and A.F.-V. were responsible for the animal experiments. L.C. and J.M.C. carried out the gene expression analyses. I.A.L, R.S., A.G.-L., L.K., and M.J.M. carried out the fecal analyses.

The authors declare that there is no duality of interest associated with this article.

5 References

[1] Gylling, H., Plat, J., Turley, S., Ginsberg, H. N. et al., Plant sterols and plant stanols in the management of dyslipidaemia and prevention of cardiovascular disease. *Atherosclerosis* 2014, *232*, 346–360.

[2] Cofan, M., Ros, E., Clinical application of plant sterol and stanol products. *J. AOAC Int.* 2015, *98*, 701–706.

[3] Calpe-Berdiel, L., Escola-Gil, J. C., Blanco-Vaca, F., New insights into the molecular actions of plant sterols and stanols in cholesterol metabolism. *Atherosclerosis* 2009, *203*, 18–31.

[4] De Smet, E., Mensink, R. P., Plat, J., Effects of plant sterols and stanols on intestinal cholesterol metabolism: suggested mechanisms from past to present. *Mol. Nutr. Food Res.* 2012, *56*, 1058–1072.

[5] Lo Sasso, G., Murzilli, S., Salvatore, L., D'Errico, I. et al., Intestinal specific LXR activation stimulates reverse cholesterol transport and protects from atherosclerosis. *Cell Metab.* 2010, *12*, 187–193.

[6] Hu, X., Steffensen, K. R., Jiang, Z. Y., Parini, P. et al., LXR-beta activation increases intestinal cholesterol absorption, leading to an atherogenic lipoprotein profile. *J. Intern. Med.* 2012, *272*, 452–464.

[7] Plat, J., Nichols, J. A., Mensink, R. P., Plant sterols and stanols: effects on mixed micellar composition and LXR (target gene) activation. *J. Lipid Res.* 2005, *46*, 2468–2476.

[8] Yang, C., Yu, L., Li, W., Xu, F. et al., Disruption of cholesterol homeostasis by plant sterols. *J. Clin. Invest.* 2004, *114*, 813–822.

[9] Kaneko, E., Matsuda, M., Yamada, Y., Tachibana, Y. et al., Induction of intestinal ATP-binding cassette transporters by a phytosterol-derived liver X receptor agonist. *J. Biol. Chem.* 2003, *278*, 36091–36098.

[10] Hoang, M. H., Jia, Y., Jun, H. J., Lee, J. H. et al., Fucosterol is a selective liver X receptor modulator that regulates the expression of key genes in cholesterol homeostasis in macrophages, hepatocytes, and intestinal cells. *J. Agric. Food Chem.* 2012, *60*, 11567–11575.

[11] Brauner, R., Johannes, C., Ploessl, F., Bracher, F., Lorenz, R. L., Phytosterols reduce cholesterol absorption by inhibition of 27-hydroxycholesterol generation, liver X receptor alpha activation, and expression of the basolateral sterol exporter ATP-binding cassette A1 in Caco-2 enterocytes. *J. Nutr.* 2013, *142*, 981–989.

[12] Jakulj, L., van Dijk, T. H., de Boer, J. F., Kootte, R. S. et al., Transintestinal cholesterol transport is active in mice and humans and controls ezetimibe-induced fecal neutral sterol excretion. *Cell. Metab.* 2016, *24*, 783–794.

[13] van der Veen, J. N., van Dijk, T. H., Vrins, C. L., van Meer, H. et al., Activation of the liver X receptor stimulates transintestinal excretion of plasma cholesterol. *J. Biol. Chem.* 2009, *284*, 19211–19219.

[14] Brufau, G., Kuipers, F., Lin, Y., Trautwein, E. A., Groen, A. K., A reappraisal of the mechanism by which plant sterols promote neutral sterol loss in mice. *PLoS One* 2011, *6*, e21576.

[15] Calpe-Berdiel, L., Escola-Gil, J. C., Blanco-Vaca, F., Phytosterol-mediated inhibition of intestinal cholesterol absorption is independent of ATP-binding cassette transporter A1. *Br. J. Nutr.* 2006, *95*, 618–622.

[16] Plosch, T., Kruit, J. K., Bloks, V. W., Huijkman, N. C. et al., Reduction of cholesterol absorption by dietary plant sterols and stanols in mice is independent of the abcg5/8 transporter. *J. Nutr.* 2006, *136*, 2135–2140.

[17] Calpe-Berdiel, L., Escola-Gil, J. C., Blanco-Vaca, F., Are LXR-regulated genes a major molecular target of plant sterols/stanols? *Atherosclerosis* 2007, *195*, 210–211.

[18] Calpe-Berdiel, L., Escola-Gil, J. C., Ribas, V., Navarro-Sastre, A. et al., Changes in intestinal and liver global gene expression in response to a phytosterol-enriched diet. *Atherosclerosis* 2005, *181*, 75–85.

[19] Llaverias, G., Escola-Gil, J. C., Lerma, E., Julve, J. et al., Phytosterols inhibit the tumor growth and lipoprotein oxidizability induced by a high-fat diet in mice with inherited breast cancer. *J. Nutr. Biochem.* 2013, *24*, 39–48.

[20] Escola-Gil, J. C., Julve, J., Llaverias, G., Urpi-Sarda, M. et al., Resveratrol administration or SIRT1 overexpression does not increase LXR signaling and macrophage-to-feces reverse cholesterol transport in vivo. *Transl. Res.* 2013, *161*, 110–117.

[21] Miettinen, T. A., Gas-liquid chromatographic determination of fecal neutral sterols using a capillary column. *Clin. Chim. Acta.* 1982, *124*, 245–248.

[22] Mosele, J. I., Gosalbes, M. J., Macia, A., Rubio, L. et al., Effect of daily intake of pomegranate juice on fecal microbiota and feces metabolites from healthy volunteers. *Mol. Nutr. Food Res.* 2015, *59*, 1942–1953.

[23] Cedo, L., Metso, J., Santos, D., Sanchez-Quesada, J. L. et al., Consumption of polyunsaturated fat improves the saturated fatty acid-mediated impairment of HDL antioxidant potential. *Mol. Nutr. Food Res.* 2015, *59*, 1987–1996.

[24] Calpe-Berdiel, L., Rotllan, N., Fievet, C., Roig, R. et al., Liver X receptor-mediated activation of reverse cholesterol transport from macrophages to feces in vivo requires ABCG5/G8. *J. Lipid Res.* 2008, *49*, 1904–1911.

[25] Wagner, B. L., Valledor, A. F., Shao, G., Daige, C. L. et al., Promoter-specific roles for liver X receptor/corepressor complexes in the regulation of ABCA1 and SREBP1 gene expression. *Mol. Cell Biol.* 2003, *23*, 5780–5789.

[26] Altmann, S. W., Davis, H. R., Jr., Zhu, L. J., Yao, X. et al., Niemann-Pick C1 like 1 protein is critical for intestinal cholesterol absorption. *Science* 2004, *303*, 1201–1204.

[27] Hayes, K. C., Pronczuk, A., Wijendran, V., Beer, M., Free phytosterols facilitate excretion of endogenous cholesterol in gerbils. *J. Nutr. Biochem.* 2005, *16*, 305–311.

[28] Rideout, T. C., Harding, S. V., Jones, P. J., Consumption of plant sterols reduces plasma and hepatic triglycerides and modulates the expression of lipid regulatory genes and de novo lipogenesis in C57BL/6J mice. *Mol. Nutr. Food Res.* 2010, *54* (Suppl 1), S7–S13.

[29] Schonewille, M., Brufau, G., Shiri-Sverdlov, R., Groen, A. K., Plat, J., Serum TG-lowering properties of plant sterols and stanols are associated with decreased hepatic VLDL secretion. *J. Lipid Res.* 2014, *55*, 2554–2561.

[30] Uchida, K., Takase, H., Nomura, Y., Takeda, K. et al., Changes in biliary and fecal bile acids in mice after treatments with diosgenin and beta-sitosterol. *J. Lipid Res.* 1984, *25*, 236–245.

[31] Altemus, J. B., Patel, S. B., Sehayek, E., Liver-specific induction of Abcg5 and Abcg8 stimulates reverse cholesterol transport in response to ezetimibe treatment. *Metabolism* 2014, *63*, 1334–1341.

[32] Plosch, T., Kok, T., Bloks, V. W., Smit, M. J. et al., Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. *J. Biol. Chem.* 2002, *277*, 33870–33877.

[33] Repa, J. J., Turley, S. D., Lobaccaro, J. A., Medina, J. et al., Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* 2000, *289*, 1524–1529.

[34] Schultz, J. R., Tu, H., Luk, A., Repa, J. J. et al., Role of LXRs in control of lipogenesis. *Genes Dev.* 2000, *14*, 2831–2838.

[35] Wang, Y., Rogers, P. M., Su, C., Varga, G. et al., Regulation of cholesterologenesis by the oxysterol receptor, LXRalpha. *J. Biol. Chem.* 2008, *283*, 26332–26339.

[36] Grefhorst, A., Elzinga, B. M., Voshol, P. J., Plosch, T. et al., Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. *J. Biol. Chem.* 2002, *277*, 34182–34190.

[37] Repa, J. J., Liang, G., Ou, J., Bashmakov, Y. et al., Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev.* 2000, *14*, 2819–2830.

[38] Mendez-Gonzalez, J., Suren-Castillo, S., Calpe-Berdiel, L., Rotllan, N. et al., Disodium ascorbyl phytostanol phosphate (FM-VP4), a modified phytostanol, is a highly active hypocholesterolaemic agent that affects the enterohepatic circulation of both cholesterol and bile acids in mice. *Br. J. Nutr.* 2010, *103*, 153–160.

[39] El Kasmi, K. C., Anderson, A. L., Devereaux, M. W., Vue, P. M. et al., Phytosterols promote liver injury and Kupffer cell activation in parenteral nutrition-associated liver disease. *Sci. Transl. Med.* 2013, *5*, 206ra137.

