Novel pathways in cholesterol metabolism to combat cardiometabolic diseases

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FXR activation resolves dyslipidemia and decreases adiposity in APOE*3-Leiden.CETP transgenic mice fed a Western-type diet

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Sumitted

Abstract

Background

The bile acid (BA) receptor farnesoid X receptor (FXR) represents a promising target for therapy of metabolic syndrome (MetS)-associated diseases. Because the effects of pharmacological modulation of BA signaling on dyslipidemia are incompletely understood, we investigated the effects of FXR activation in a humanized mouse model of MetS.

Methods and results

Male (12-14 weeks old) APOE*3-Leiden.CETP-transgenic mice were fed a Western-type diet (WTD) for 8 weeks to induce MetS before treatment with the non-steroidal FXR-agonist PX20606 (PX, 10 mg/kg/day, mixed into WTD) for 4 weeks. Food intake and adiposity were reduced in PX-treated mice compared to controls, whereas glucose tolerance was improved. PX treatment profoundly decreased plasma triglyceride (TG) (0.77 vs. 1.51 mmol/L in controls, $p<0.001$) and cholesterol (2.4 vs. 9.2 mmol/L in controls, $p<0.001$) levels, primarily due to reductions of ApoB-containing lipoproteins. Consistent with a strong reduction of hepatic Cyp8b1 expression, tauro-β-muricholic acid became the most abundant BA in bile of PX-treated mice. The more hydrophilic BA pool in PX-treated mice was associated with impaired intestinal cholesterol absorption and strongly increased fecal cholesterol excretion. Furthermore, intestinal fat absorption was substantially reduced upon PX treatment. In addition, increased ApoC2/ApoC3 ratios in plasma suggest augmented clearance of VLDL-TG in PX-treated mice.

Conclusions

FXR stimulation by PX20606 enhances fecal cholesterol disposal and corrects dyslipidemia in WTD-fed APOE*3-Leiden.CETP-transgenic mice. Although care should be taken when extrapolating these results to humans, particularly because of species-differences in BA metabolism, pharmacological modulation of BA metabolism may hold potential for the treatment of MetS-associated dyslipidemia.

Introduction

Metabolic syndrome (MetS) and its co-morbidities, including non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), type 2 diabetes (T2D) and cardiovascular diseases (CVD), represent a growing burden to healthcare systems across the globe. Although considerable progress has been made in our understanding of these MetS-associated morbidities in the last decades, currently available therapies only partially reduce the risk of MetS-associated complications like CVD. With the introduction of statins and, more recently, PCSK9-inhibitors, hypercholesterolemia can be relatively well-controlled [1]. However, a considerable residual risk of CVD remains in MetS patients despite the fact that their plasma cholesterol levels are maintained within the normal range [2]. Hypertriglyceridemia may represent an independent risk factor for CVD [3, 4]. Currently available cholesterol-lowering drugs also impact plasma triglyceride (TG) levels. However, the effects of the most commonly used cholesterol-lowering agents, such as statins, ezetimibe and PCSK9 inhibitors, on plasma TG are limited. Drugs with more pronounced effects on plasma TG levels are available, such as niacin and fibrates, but their impact on CVD risk when added to low density lipoprotein (LDL)-lowering therapy is questioned [5]. Hence, additional treatment strategies are required to improve the health perspectives of individuals with MetS.

One of the emerging strategies to improve MetS-related metabolic derangements is to interfere in bile acid (BA) signaling pathways. In the last two decades, BAs have emerged as important signaling molecules that activate various nuclear and membrane-bound receptors, including farnesoid X receptor (FXR) [6, 7], Takeda G protein-coupled receptor 5 (TGR5, GPBAR1) [8], vitamin D receptor (VDR) [9] and sphingosine-1-phosphate receptor 2 (S1PR2) [10], and thereby exert hormone-like actions. In addition to its well-known role in controlling BA homeostasis, FXR has also been shown to play a prominent role in the regulation of glucose, lipid and lipoprotein metabolism [11]. Agonistic ligands of the BA receptor FXR have been developed for the treatment of cholestatic liver diseases, but may also hold potential for the treatment of NAFLD/NASH [12] and for improving glucose metabolism in T2D [13].

In mice, FXR activation decreases plasma TG and total cholesterol [14], whereas whole-body and liver-specific FXR-deficiency is associated with higher plasma TG and cholesterol levels [15, 16]. Data obtained from clinical trials with synthetic agonistic ligands for FXR suggest a more complex relation between this nuclear receptor and plasma lipids in humans. The steroidal FXR agonist obeticholic acid (OCA, INT-747) has been shown to induce an increase of plasma LDL-cholesterol (LDL-C) levels and a temporal decrease of plasma TG levels in NASH patients [17, 18], while the non-steroidal FXR agonist Cilofexor (GS-9674) did not affect plasma lipids in individuals with NASH [19].

Mechanistic studies in mice have identified multiple pathways through which FXR modulates lipid and lipoprotein metabolism. Hepatic gene expression analyses suggest that FXR activation may decrease de novo lipogenesis (DNL) through inhibition of sterol regulatory element binding transcription factor 1c (Srebf1c) expression [20, 21], but conclusive evidence that FXR indeed inhibits DNL, e.g., by direct measurements of DNL using in vivo fluxomics studies with stable isotopes, is still lacking. FXR-mediated downregulation of microsomal triglyceride transfer protein (Mttp) expression [22] may result in lower lipidation rates of apolipoprotein B (ApoB) and reduce secretion of TG within very low-density lipoprotein (VLDL) particles by the liver. In addition, FXR activation may increase VLDL catabolism

via modulation of the hepatic expression of Apoc2 [23], Apoc3 [24] and angiopoietin-like 3 (Angptl3) [20]. The reduction of plasma cholesterol upon FXR stimulation in wild-type (WT) mice is mainly explained by a decrease in high density lipoprotein-cholesterol (HDL-C), likely due to inhibition of Apoa1 expression [25] and upregulation of scavenger receptor class B, member 1 (Scarb1) [26].

Lipoprotein metabolism in mice differs considerably from humans. Whereas in humans (V)LDL carry about two third of plasma cholesterol, in mice the vast majority of plasma cholesterol is carried by HDL [27]. These differences are caused by a rapid clearance of TG-rich lipoprotein remnants as well as by the absence of the cholesteryl ester transfer protein (CETP) in mice. CETP mediates transfer of cholesteryl esters (CE) from HDL to VLDL and thereby decreases the HDL-C/VLDL-C ratio in humans [28, 29]. APOE*3-Leiden.CETP transgenic mice [30] express human APOE*3-Leiden, causing attenuated clearance of TG-rich lipoprotein remnants, as well as human CETP. When fed a Western-type diet (WTD), these mice display a lipoprotein distribution resembling the dyslipidemic profile that is characteristic for humans with MetS, i.e., elevated plasma TG and markedly higher (V)LDL-C [30]. In contrast to WT mice, APOE*3-Leiden.CETP transgenic mice react to hypolipidemic drugs in a similar way as humans do [31]. Furthermore, we recently reported that the progression of MetS in these mice during a long term high-fat diet (HFD) challenge bears strong resemblance with MetS development in humans [32]. Hence, APOE*3-Leiden.CETP transgenic mice represent an attractive model to address the effects of pharmacological FXR modulation on MetS-associated dyslipidemia.

In the current study, we assessed the effects of pharmacological FXR activation on lipid and lipoprotein metabolism in APOE*3-Leiden.CETP transgenic mice which had been pre-treated with a WTD for 8 weeks. We found that treatment with a non-steroidal FXR agonist decreased adiposity and substantially lowered plasma TG and cholesterol levels, mainly due to a reduction of ApoB-containing lipoproteins. FXR stimulation caused a more hydrophilic BA pool which was associated with impaired intestinal lipid absorption. Analysis of hepatic gene expression and the plasma proteome revealed that the reduction of plasma TG upon FXR stimulation is likely mediated through changes in hepatic production of factors that modulate lipoprotein lipase (LPL)-mediated clearance of TG-rich lipoproteins.

Methods

Animal experiments

Hyperlipidemic male APOE*3-Leiden.CETP transgenic mice [30] were individually housed in a light- (12 hours light/12 hours dark) and temperature-controlled (21°C) facility. Only mice with fasting (5 hours) plasma TG levels >1 mmol/L at the age of 12 weeks, while still fed a standard laboratory chow diet (RMH-B, AB Diets, Woerden, The Netherlands), were included in the experiments. These mice were then fed a WTD containing 60% (calories) fat and 0.25% (w/w) added cholesterol (D14010701, Research Diets, New Brunswick, NJ, USA). When stated, mice received 10 mg/kg/day of the FXR agonist PX20606 (PX; Phenex Pharmaceuticals, AG, Heidelberg, Germany), which was mixed into the WTD. A first cohort of

animals (n=9 mice/group) was used to study the effects of pharmacological FXR stimulation on body weight, plasma lipids, intestinal fat absorption, DNL, cholesterol synthesis and VLDL-TG production, while glucose tolerance, fractional cholesterol absorption and bile formation were studied in a second cohort of animals (control, n=9; PX, n=8 mice/group). Experiments were conducted in conformity with the law on the welfare of laboratory animals, and experimental procedures were approved by the responsible ethics committees of the universities of Groningen and Leiden.

Experimental procedures

Starting from the age of 12-14 weeks, all mice that were included in the experiments were fed WTD ad libitum for 8 weeks. Next, animals were matched for body weight and plasma lipids and allocated to either the treatment or the control group. Treated mice received WTD with added PX for 4 weeks, while control animals were maintained on the WTD without any addition. Just before start of PX treatment and 4 weeks later, blood was collected from the orbital plexus after 5 hours fasting (08:00-13:00 h). Feces was collected continuously for 72 hours, just before the start of treatment as well as 2 and 4 weeks thereafter. A glucose tolerance test was performed at 2.5 weeks after onset of PX treatment. After 5 hours of fasting (08:00-13:00 h), mice received a glucose bolus (1.25 g/kg) by intraperitoneal (i.p.) injection. Blood glucose levels were measured at t=0, 15, 30, 60, 90 and 120 minutes after glucose injection using a handheld glucose meter (Onetouch Ultra, LifeScan Benelux, Beerse, Belgium). To determine fractional cholesterol absorption, mice received an intravenous (i.v.) dose of 0.3 mg D5-cholesterol dissolved in intralipid (20%, pharmacy UMCG, Groningen, The Netherlands) and an oral dose of 0.6 mg D7-cholesterol dissolved in medium-chain TG oil 10 days before the end of the experiment [33]. Blood spots collected from the tail at 0, 3, 6, 12, 24, 48, 72, 96, 120, 144, and 168 hours after bolus administration were used to determine enrichments of the administered cholesterol tracers in the blood circulation using gas chromatography-mass spectrometry (GC-MS). Fractional cholesterol absorption was calculated from the areas under the curves of appearance of the orally and i.v. administered cholesterol tracers in blood, corrected for the administered dosages as described [33]. Intestinal lipid absorption efficiency was determined using the fat balance method [34]. The fat absorption coefficient was calculated by subtracting the fecal fat output from the fat intake, divided by the fat intake, multiplied by 100%. To determine hepatic DNL, mice received 2% [1-13C]acetate in the drinking water starting from 3 days before sacrifice. Lipogenesis was determined using mass isotopomer distribution analysis (MIDA), as detailed elsewhere [35]. In part of the animals, the gallbladder was cannulated directly following ligation of the common bile duct under Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg) anesthesia. Subsequently, these mice were placed in a humidified incubator to maintain body temperature during bile collection (30 minutes) [36]. At the end of the experiment, the animals were sacrificed by cardiac puncture, tissues were removed and snap-frozen in liquid nitrogen.

In another cohort of animals, VLDL-TG production was measured following inhibition of LPL activity by i.p. injection with poloxamer 407 (1000 mg/kg body weight) [37]. After 0, 30, 60, 120 and 240 minutes small (~25 μL) blood samples were collected by tail bleeding for measurement of TG. VLDL-TG production was calculated from the slope of the increasing TG concentrations between 60 and 240 minutes as described [38].

Analytical procedures

Plasma TG and cholesterol were measured according to standard procedures using commercially available reagents (Roche Diagnostics, Basel, Switzerland and DiaSys Diagnostic Systems, Holzheim, Germany). Plasma insulin was measured by ELISA (Alpco Diagnostics, Salem, NH, USA) according to the manufacturer's instructions. Hepatic lipids were extracted according to Bligh & Dyer [39], dried and redissolved in 2% Triton-X100. TG and cholesterol were subsequently quantified using the commercial reagents listed above. BAs in bile were quantified using liquid chromatography (LC)-MS/MS [40], whereas BAs in feces were measured using capillary GC as described [41]. The hydrophobicity index of biliary BAs was calculated according to Heuman [42]. Cholesterol in bile and neutral sterols, i.e., cholesterol and its bacterial derivatives, in feces were trimethylsilylated and measured by GC as detailed elsewhere [41]. Biliary phospholipids were extracted [39] and measured as described [43].

Gene expression analysis

Hepatic RNA was extracted using TRI-Reagent (Sigma, St. Louis, MO) and 1 μg was reverse transcribed using Moloney–Murine Leukemia Virus (M-MLV) reverse transcriptase (Life Technologies, Bleiswijk, The Netherlands). Real-time quantitative polymerase chain reaction (qPCR) was performed on a QuantStudio-3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using Taqman primer-probe combinations. Data were normalized to cyclophilin as a housekeeping gene and further normalized to the mean of the control group.

Targeted quantitative proteomics

Proteins in plasma were quantified by targeted proteomics using concatemers of isotopically-labeled standard peptides (see major resources table) containing 13C-labeled lysines and arginines (Polyquant GmbH, Germany) essentially as described [36]. Briefly, after reduction with 10 mmol/L dithiothreitol and alkylation with 55 mmol/L iodoacetamide, in-gel digestion was performed on 1 μL plasma plus 20 ng isotopically-labeled concatemers using trypsin (1:100 g/g sequencing grade modified trypsin V5111; Promega). Solid-phase extraction (SPE C18-Aq 50 mg/mL, Gracepure, Thermo Fisher Scientific, Waltham, MA, USA) was then performed for sample clean-up after which the peptides were separated by LC on a nano-ultra high performance liquid chromatography (UHPLC) system (Ultimate UHPLC focused; Dionex, Thermo Fisher Scientific) and analyzed by a triple quadrupole MS equipped with a nano-electrospray ion source (TSQ Vantage; Thermo Fisher Scientific). For the LC-MS measurements, an amount of digested peptides equivalent to a total protein amount within 100 nL plasma was injected. The concentrations of endogenous peptides were calculated using the known concentrations of the labeled standard peptides.

Statistics

Statistical significance of differences between groups was assessed using the Mann-Whitney U test unless stated otherwise. Body weights after onset of PX treatment were compared to body weights before PX treatment using repeated measurements ANOVA. Differences between groups were considered significant when $p<0.05$.

Results

FXR stimulation decreases adiposity and dyslipidemia in WTD-fed APOE*3-Leiden. CETP transgenic mice

MetS was induced in APOE*3-Leiden.CETP transgenic mice by feeding them a WTD for 8 weeks. During this period, the animals gained considerable body weight (**Fig. 1A**). Mice were then assigned to either the FXR agonist treatment group or the control group based on their body weight and plasma lipid levels to ensure that these parameters were similar in both groups at the start of the intervention. Mice receiving PX started to steadily lose body weight ($p<0.001$) while body weight of untreated animals plateaued (**Fig. 1A**). Food intake, measured continuously from week 2-4 after onset of PX treatment, was decreased in treated animals (-14% over this 3-week period; 2.29 vs. 2.66 g/day, $p<0.001$) (**Fig. 1B**). The difference in food intake between groups became somewhat smaller over time, but in the last week of treatment the difference in food intake was still significant (-8%; 2.54 vs. 2.77 g/day, $p=0.037$).

In line with previous observations [32], control WTD-fed APOE*3-Leiden.CETP transgenic mice had high plasma TG and cholesterol levels and displayed a lipoprotein profile resembling the profile observed in dyslipidemic MetS patients, with high (V)LDL (**Fig. 1C, D and Fig. S1A**). PX treatment markedly improved the plasma lipid profile, as it decreased plasma TG levels (-50%; $p<0.001$, **Fig. 1C**) as well as cholesterol levels (-75%, $p<0.001$, **Fig. 1D**). Moreover, FPLC-profiling revealed strong decreases in (V)LDL-C, leading to a substantially improved HDL-C/(V)LDL-C ratio (**Fig. 1C, D and Fig. S1A**). In fact, the dyslipidemic lipoprotein profile that is characteristic for WTD-fed APOE*3-Leiden.CETP transgenic mice, was normalized by PX treatment (see **Fig. S1B** for a typical FPLC cholesterol profile of chow-fed C57BL/6J mice).

The liver plays a central role in the regulation of plasma lipoprotein metabolism. Therefore, we measured liver weight and hepatic lipid content. In line with previous observations in WT mice [36], liver weights tended to be higher in PX-treated animals compared to controls (**Fig. S2A**), but hepatic TG and cholesterol contents were not significantly affected (**Fig. S2B, and C**). The changes in body weight did translate into modest effects on glucose homeostasis. Fasting blood glucose levels tended to be lower in PX-treated mice compared to controls ($p=0.054$, **Fig. 2A**), while fasting plasma insulin levels were similar between groups (**Fig. 2B**). Furthermore, a glucose tolerance test demonstrated improved glucose handling in PX-treated mice (**Fig. 2C**).

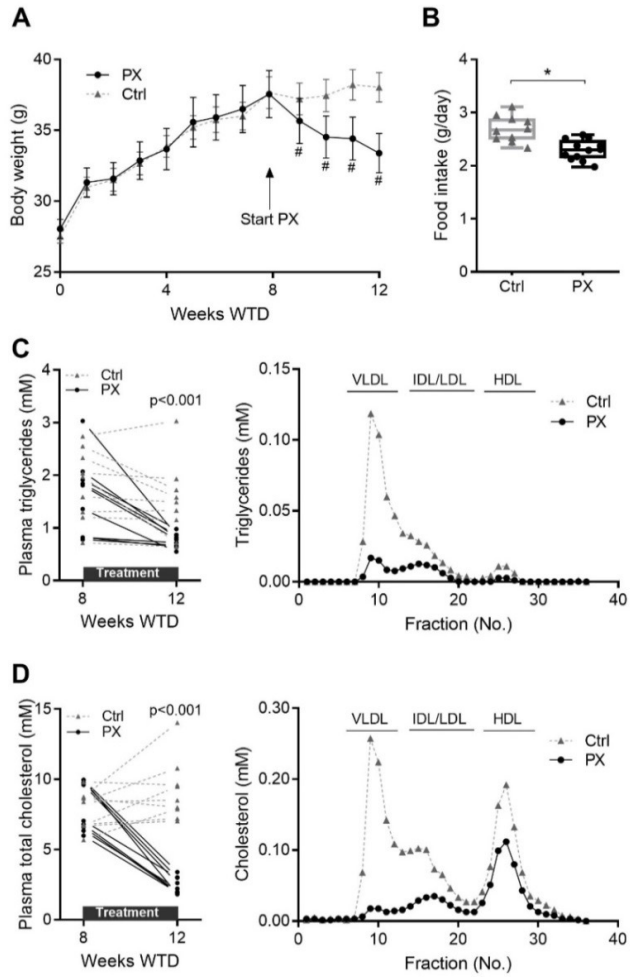

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Figure 1. FXR stimulation reduces body weight, food intake, and plasma lipids. (A) Body weight of Western-type diet-fed APOE*3-Leiden.CETP mice before and during treatment without or with PX20606 (PX). (B) Average weekly food intake. (C) Triglycerides and (D) total cholesterol in plasma before and after 4 weeks of PX treatment as well as in FPLC fractions of pooled plasma samples that were collected at the end of treatment. #Significantly different from body weight of the same group at the start of PX treatment (week 8) by repeated measurements ANOVA followed by Bonferroni correction for multiple comparisons. *Significant difference between groups by Mann-Whitney U test. Panels A, C, D, n=9 animals/group. Data in panel B is obtained from a different cohort of n=10 (Ctrl) and n=12 (PX) animals/group. Ctrl, Control; PX, PX20606; WTD, Western-type diet.

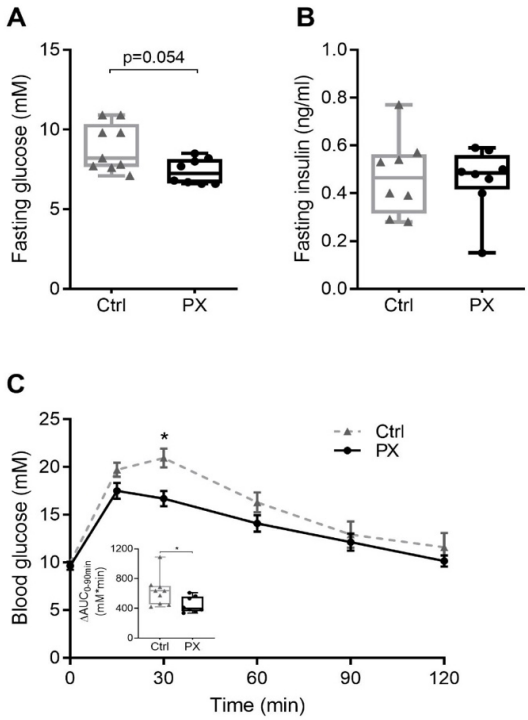

Figure 2. FXR stimulation improves glucose handling. (A) Fasting blood glucose and (B) fasting plasma insulin concentrations in Western-type diet-fed APOE*3-Leiden.CETP transgenic mice treated with PX20606 (PX) or control diet for 4 weeks, and (C) glucose concentrations during an intraperitoneal glucose tolerance test that was performed after 2.5 weeks of PX treatment in the same animals. Insert, area under the incremental curve from 0 to 90 minutes after glucose injection. *Significant difference between groups, n=9 (Ctrl) and n=8 (PX) animals/group. Ctrl, Control; PX, PX20606.

PX treatment increases hydrophilicity of BA pool and decreases intestinal lipid absorption

A major function of FXR is to maintain BA homeostasis by limiting BA production. Hepatic mRNA expression levels of Cyp7a1, Cyp8b1 and Cyp7b1 were lower in mice treated with PX (**Fig. 3A**), strongly suggesting that BA synthesis was indeed reduced in these mice. In line with the effects on gene expression, fecal BA excretion, reflecting hepatic BA production under steady-state conditions, was profoundly decreased in PX-treated animals compared to controls already after 2 weeks of PX treatment and was maintained at a similar level thereafter (**Fig. 3B**). Gallbladder cannulations were performed to assess the effects of PX-treatment on bile formation in the obese dyslipidemic APOE*3-Leiden.CETP transgenic mice. Bile flow was about 2-fold higher in mice treated with PX compared to controls, whereas neither total biliary BA nor biliary phospholipid secretion were significantly affected by treatment with the FXR agonist (**Fig. 3C-E**). Biliary cholesterol concentrations were similar in both groups (data not shown), but biliary cholesterol secretion rates were ~1.8-fold higher upon PX treatment (**Fig. 3F**) due to increased bile flow.

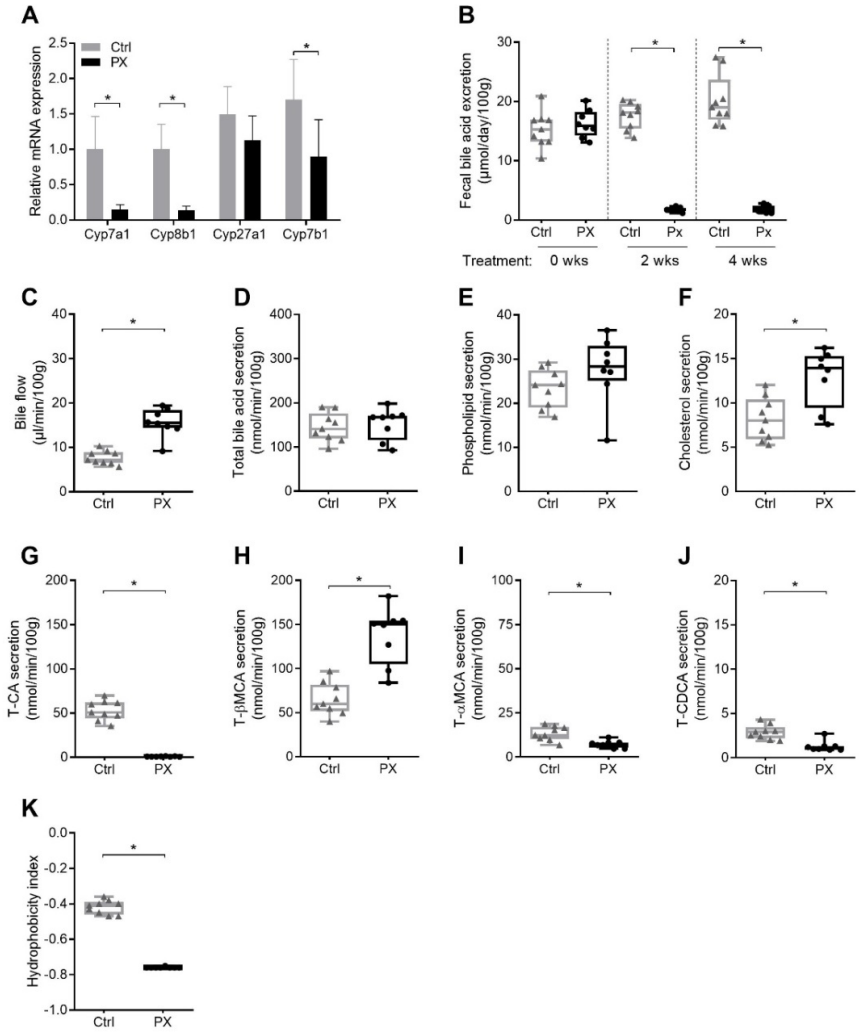

Figure 3. FXR stimulation reduces biliary secretion of taurocholic acid, but increases secretion of tauro-β-muricholic acid. (A) Hepatic mRNA expression of genes involved in bile acid synthesis in Western-type diet (WTD)-fed APOE*3-Leiden.CETP transgenic mice treated with PX20606 (PX) or controls. (B) Fecal bile acid excretion, (C) bile flow, (D) biliary bile acid, (E) phospholipid and (F) cholesterol secretion rates determined after gallbladder cannulation. (G-J) Secretion rates of individual bile acid species into the bile. (K) Hydrophobicity index of biliary bile acids. *Significant difference between groups, n=9 (Ctrl) and n=8 (PX) animals/group. Ctrl, Control; PX, PX20606; T-CA, taurocholic acid; T-αMCA, Tauro-αmuricholic acid; T-βMCA, tauro-βmuricholic acid; T-CDCA, taurochenodeoxycholic acid.

Because not only the total amounts of BAs, but also the physicochemical properties of the BAs secreted into the bile are important determinants of bile formation and intestinal lipid handling, we also quantified the effects of PX treatment on biliary secretion rates of the individual BA species. In contrast to lack of effect of PX on the total biliary BA secretion rate, striking differences became apparent when the secretion rates of individual BA species into the bile were assessed. Taurocholic acid (T-CA) was abundant in bile of untreated animals, but biliary T-CA secretion dropped to negligible amounts upon PX treatment (**Fig. 3G**). Instead, tauro-βmuricholic acid (T-βMCA) became by far the most abundant BA in bile of PX-treated mice, while T-αMCA and taurochenodeoxycholic acid (T-CDCA) were secreted into bile at lower rates (**Fig. 3H-J**). Due to the altered BA composition, the hydrophobicity of biliary BAs was substantially decreased upon PX treatment (**Fig. 3K**).

In line with previous observations that activation of FXR by PX strongly stimulates cholesterol disposal by increasing transintestinal cholesterol excretion (TICE) in chow-fed C57BL/6J mice [36], the PX-treated WTD-fed mice in the current study showed a 3-fold higher fecal neutral sterol excretion compared to controls (**Fig. 4A**) despite similar cholesterol intake (**Fig. 4B**). The hydrophilic BA pool in mice that were treated with PX was associated with substantially decreased fractional absorption of cholesterol from the intestine (10% vs. 42%, **Fig. 4C**). The alterations in sterol fluxes induced by PX treatment impacted the whole-body sterol balance of the mice, calculated as dietary cholesterol intake minus fecal loss of cholesterol and bile acids (**Fig. 4D**). As would be expected in an atherogenic mouse model, non-treated APOE*3-Leiden.CETP transgenic mice displayed a mildly positive sterol balance, indicating accumulation of cholesterol in the body. In contrast, PX-treated mice had a clear negative sterol balance, meaning that sterol loss exceeded sterol intake in these animals. Consequently, they would need to synthesize cholesterol endogenously to maintain their sterol pools. Indeed, hepatic mRNA expression of Hmgcr (**Fig. 4E**) was substantially increased upon PX treatment. Furthermore, direct measurement of cholesterol synthesis by incorporation of the precursor [1-13C]acetate into cholesterol molecules confirmed that virtually no cholesterol was synthesized in control WTD-fed APOE*3-Leiden.CETP transgenic mice, whereas endogenous cholesterol synthesis was clearly induced upon PX treatment (**Fig. 4F**). Despite these marked differences in cholesterol fluxes between groups, hepatic mRNA expression of LDL receptor (Ldlr) remained unchanged upon PX treatment. Expression of the HDL-CE selective uptake receptor Scarb1 was increased in PX-treated animals compared to controls (**Fig. 4E**). Although BAs are more important for intestinal absorption of cholesterol than for absorption of fatty acids from the intestine [44], the strong effects of the hydrophilic BA pool in PX-treated WTD-fed APOE*3-Leiden.CETP transgenic mice on cholesterol absorption let us question whether PX treatment also impacted intestinal fat absorption. Indeed, absorption of dietary lipids from the intestine dropped from 91% in control animals to 62% in mice that were treated with the FXR-agonist (**Fig. 4G**).

Decreased plasma TG upon FXR activation is not due to decreased lipogenesis or VLDL-TG production

To identify the mechanisms involved in the correction of the dyslipidemic lipoprotein profile in APOE*3-Leiden.CETP transgenic mice upon treatment with the FXR agonist, we first assessed whether hepatic DNL was reduced. Reduced hepatic mRNA expression of Srebf1c and stearoyl-coenzyme A desaturase 1 (Scd1) (**Fig. 5A**) provided indications for a reduction of fatty acid synthesis upon PX treatment. Hepatic expression of fatty acid synthase (Fasn)

was, however, not impacted by PX treatment (**Fig. 5A**). In line with the reduced expression of Scd1, direct quantification of DNL following administration of the fatty acid precursor [1-13C]acetate to the mice (**Fig. 5B-E**) demonstrated a reduction in the synthesis of palmitoleic acid (**Fig. 5C**), which is a minor fatty acid in the murine liver. Fractional synthesis of palmitic (**Fig. 5B**) and stearic acid (**Fig. 5D**) was, however, increased upon PX treatment, whereas the fraction of newly synthesized oleic acid (**Fig. 5E**), a major fatty acid found in fatty livers due to its preferential incorporation into TG molecules, was low in both groups. These data indicate that DNL is not substantially affected by FXR activation in APOE*3-Leiden.CETP transgenic mice.

Because hepatic VLDL production is an important determinant of plasma TG levels, we next examined whether hepatic VLDL-TG production was altered in APOE*3-Leiden.CETP transgenic mice upon FXR stimulation. VLDL-TG production rates did, however, not differ between treated and non-treated animals (**Fig. 5F**).

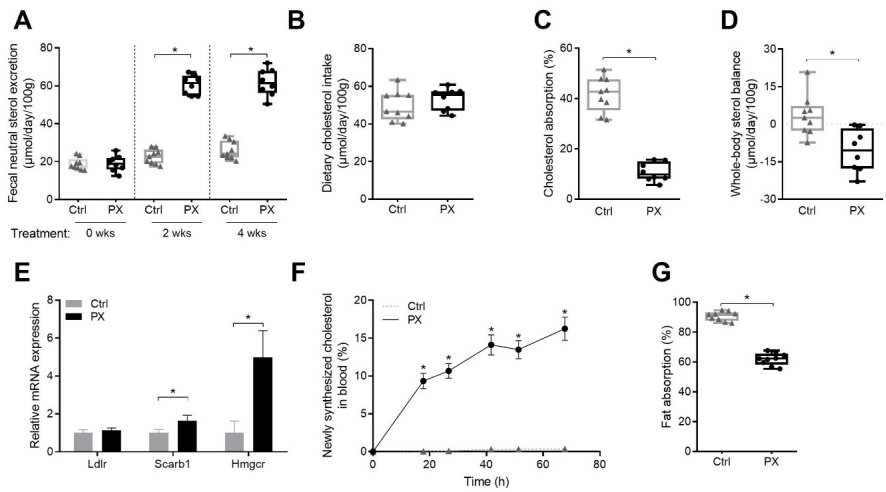

Figure 4. FXR stimulation reduces intestinal lipid absorption. (A) Fecal neutral sterol excretion, (B) dietary cholesterol intake and (C) fractional cholesterol absorption in Western-type diet (WTD)-fed APOE*3-Leiden.CETP transgenic mice treated with PX20606 (PX) or controls. (D) Whole body sterol balance calculated as cholesterol intake – (fecal neutral sterols + fecal bile acids). (E) Hepatic mRNA expression of genes involved in cholesterol metabolism. (F) Cholesterol synthesis determined by the incorporation of ^{13}C-labelled acetate into cholesterol molecules. (G) Intestinal absorption of dietary fat. *Significant difference between groups. Panels A-E, n=9 (Ctrl) and n=8 (PX) animals/group. Data in panels F and G are derived from a separate cohort of n=9 (Ctrl) and n=9 (PX) animals/group. Ctrl, Control; PX, PX20606.

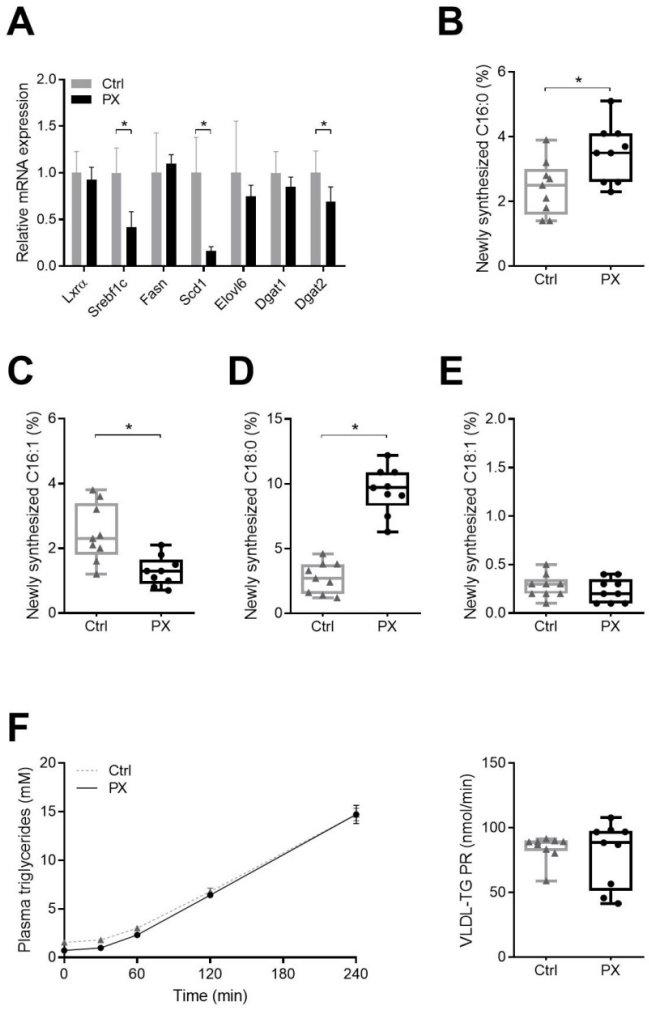

Figure 5. FXR stimulation does not quantitatively modulate *de novo* lipogenesis. (A) Hepatic mRNA expression of genes involved in fatty acid synthesis and triglyceride (TG) formation, (B-E) fractions of *de novo* synthesized fatty acids in livers, and (F) VLDL-TG production in Western-type diet-fed APOE*3-Leiden.CETP transgenic mice treated with PX20606 (PX) and controls. *Significant difference between goups. Panel A, n=9 (Ctrl) and n=8 (PX) animals/group. Panels B-F, n=9 (Ctrl) and n=9 (PX) animals/group. Ctrl, Control; PX, PX20606.

Plasma proteome analysis suggests increased VLDL-TG clearance upon FXR stimulation

The strongly reduced plasma TG levels combined with unaffected VLDL-TG production rates in the PX-treated mice, suggested that TG hydrolysis should be increased upon PX treatment. Interestingly, hepatic mRNA expression of Apoc2 was upregulated, while Apoc3 expression

remained unchanged (**Fig. 6A**), suggesting that clearance of endogenous VLDL particles may indeed be impacted by PX treatment. Therefore, we next measured a panel of plasma (apolipo) proteins that may impact lipoprotein metabolism using a targeted proteomics approach (**Fig. 6B**). In line with the decreased levels of HDL-C and LDL-C in PX-treated mice, plasma concentrations of ApoA1 and ApoB were significantly lower in these animals, while ApoE, LCAT and CETP were reduced as well. Several LPL-inhibiting factors, i.e., ApoC1, ApoC3 and ANGPTL3 were substantially decreased in the treated mice, whereas levels of LPL-stimulating ApoC2 were only modestly reduced. Interestingly, the ApoC2/ApoB ratio was increased upon PX treatment (**Fig. 6C**), whereas the ApoC3/ApoB ratio was decreased (**Fig. 6D**). Furthermore, the altered balance between LPL-activating ApoC2 and the LPL-inhibiting apolipoproteins ApoC1 and ApoC3 (**Fig. 6E, F**) suggests augmented hydrolysis of TG contained within endogenous VLDL particles upon treatment with the FXR agonist.

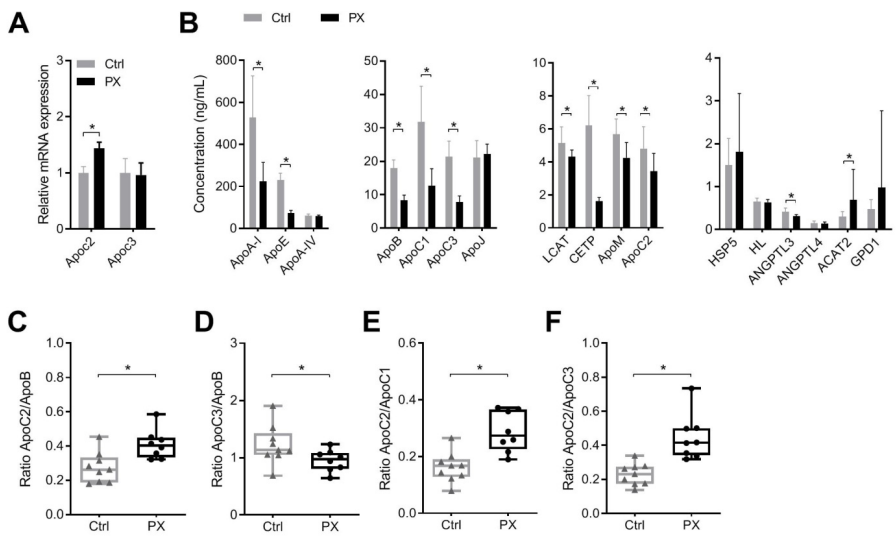

Figure 6. FXR stimulation conceivably increases VLDL catabolism by impacting on expression of lipoprotein lipase activity-modulating lipoprotein-associated proteins. (A) Hepatic mRNA expression of Apoc2 and ApoC3 in Western-type diet-fed APOE*3-Leiden. CETP transgenic mice treated with PX20606 (PX) and controls. (B) Levels of (apolipo) proteins in plasma determined by quantitative targeted proteomics. Ratios of (C) ApoC2 to ApoB, (D) ApoC3 to ApoB, (E) ApoC2 to ApoC1 and (F) ApoC2 to ApoC3 in plasma. *Significant difference between groups, n=9 (Ctrl) and n=8 (PX) animals/group. Ctrl, Control; PX, PX20606.

Discussion

In the current study, we demonstrate that pharmacological FXR stimulation with the non-steroidal FXR agonist PX cures dyslipidemia and reduces adiposity in a humanized mouse model of MetS, i.e., WTD-fed APOE*3-Leiden.CETP transgenic mice. Reduced food intake and decreased intestinal lipid absorption likely represent major causes for the observed metabolic improvements. Moreover, a shift in the balance between lipoprotein-associated

LPL-activating and –inhibiting factors conceivably contributed to the improved lipoprotein profile upon PX treatment.

In our experiments, mice were pre-fed with a WTD for 8 weeks to induce MetS before treatment with the FXR agonist was initiated. This approach was chosen to mimic the human situation, in which treatment is usually initiated in patients with already established MetS. Although the mice were treated with the FXR agonist for only 4 weeks, marked improvements of adiposity and dyslipidemia were observed. Intestinal fat absorption was markedly reduced upon treatment with PX, but this did not translate into an increase in food intake. Food intake measurements even revealed an intriguing reduction in food intake in the PX-treated animals. While this manuscript was in preparation, Higuchi et al. [45] reported that Cyp8b1-deficient mice display mild hypophagia, which is mediated via increased activation of the fat receptor GRP119 in the distal small intestine. In analogy to Cyp8b1-KO mice, FXR stimulation in APOE*3-Leiden.CETP transgenic mice resulted in

inhibition of the production of 12α-hydroxylated BAs and, consequently, a hydrophilic BA pool. Also in line with our observations in the current study, Cyp8b1-/- mice display impaired fat absorption from the intestine [46], leading to increased quantities of lipids reaching the distal small intestine where GPR119 is highly expressed. Hence, it is highly conceivable that ileal GPR119 was more activated in PX-treated mice due to higher luminal lipid concentrations. This may subsequently have slowed down gastric emptying [45] and, consequently, reduced food consumption. Although direct translation of the results obtained in the APOE*3-Leiden.CETP transgenic mice to humans is not straightforward due to fundamental differences in BA metabolism [47], the results obtained in our current study indicate that interventions leading to a more hydrophilic BA composition could have beneficial effects on body weight in MetS patients. In support of this hypothesis, treatment with the hydrophilic BA UDCA resulted in a discernible trend towards improved weight loss in obese NASH patients compared to placebo in a small clinical trial [48].

The impact of FXR stimulation on the plasma lipoprotein profile of the mice was striking. WTD-fed APOE*3-Leiden.CETP transgenic mice are often used to study interventions impacting on plasma lipid levels because their lipoprotein metabolism closely resembles that of humans with MetS. In the current study, the mice indeed displayed a pro-atherogenic lipoprotein profile. Upon treatment with the FXR agonist however, lipoprotein distribution became similar to that of chow-fed C57BL/6J mice (compare Fig. 1D with Fig. I-B of the data supplement), demonstrating that the dyslipidemia was completely cured. In line with our data, FXR stimulation has previously been shown to decrease plasma TG in C57BL6/J mice and CETP transgenic/Ldlr-/- [36, 49]. In the present study, for the first time we systemically evaluated the effects of pharmacological FXR activation on lipid and lipoprotein metabolism in a mouse model with humanized lipoprotein metabolism. FXR activation did not affect VLDL-TG production in APOE*3-Leiden.CETP transgenic mice, suggesting that enhanced clearance causes the decrease in plasma TG. Indeed, analysis of LPL activity-influencing factors in plasma revealed increased ApoC2/ApoC1 and ApoC2/ApoC3 ratios as well as decreased ANGPTL3 concentrations in PX-treated animals, suggesting increased LPL-mediated lipolysis of VLDL-TG. These observations are in agreement with the studies on FXR-agonism in other mouse models [20, 24, 50]. Watanabe et al. reported downregulation of hepatic Angptl3 mRNA expression in C57BL/6J mice upon 1 day of CA feeding via a SHP-dependent mechanism [20]. Similarly, Claudel et al. reported a reduction of hepatic Apoc3 mRNA as well as serum ApoC3

concentrations in mice that had received 0.5% TCA in their diets for 5 days [24]. Displacement of HNF4α from a direct repeat (DR1) site in the APOC3 gene promoter was suggested to be responsible for the decreased APOC3 expression upon FXR activation in mice as well as in human hepatocytes [24]. In addition, Jadhav et al. observed upregulation of hepatic Apoc2 mRNA in response to INT-767 treatment, a TGR5/FXR-double agonist, in Apoe-/- mice [51]. Collectively, these data suggest that FXR stimulation reduces plasma TG by accelerating VLDL-TG catabolism.

FXR activation by PX also robustly reduced plasma cholesterol levels in APOE*3-Leiden. CETP transgenic mice. Although HDL-C was reduced to a moderate extent, decreased plasma cholesterol levels upon PX treatment were primarily attributable to a reduction of ApoB-containing lipoproteins. Hepatic expression of Ldlr was not affected by PX treatment. Hence, other factors appear to be the main drivers of the improved lipoprotein profile. We have demonstrated previously that PX induces a rerouting of cholesterol fluxes in chow-fed C57BL/6J mice by increasing its removal via the TICE pathway, leading to strongly increased fecal cholesterol disposal [36]. A similar effect was noticed in the present study. Although the effects appear to be somewhat masked by the high cholesterol content of the diet that was used, PX induced a considerable increase in fecal cholesterol loss, which was compensated in part by enhanced de novo synthesis. We speculate this rerouting of cholesterol fluxes together with a decreased reabsorption of transintestinally secreted cholesterol [50] to be a main mechanism contributing to normalization of the lipid profile in the APOE*3-Leiden.CETP transgenic mice. The effect might have been enhanced by decreased activity of CETP in these mice upon PX treatment. CETP transfers cholesteryl esters from HDL towards VLDL in exchange for TG [28, 29]. Endogenous CETP activity strongly increases with plasma TG levels [52] and was therefore likely high in our untreated WTD-fed fed mice. Upon PX treatment however, CETP expression as well as plasma TG concentrations dropped substantially, likely resulting in a strong decrease in net cholesteryl ester transfer from HDL to VLDL and, thus, in reduced (V) LDL-C levels and improved HDL-C/(V)LDL-C ratios. Additionally, increased hepatic uptake of triglyceride-rich lipoprotein-remnants due to accelerated TG hydrolysis upon PX treatment may have contributed to the lower plasma cholesterol levels observed in these animals [53]. The modest reduction in HDL-C that was observed in PX-treated mice conceivably results from the reduced expression of Apoa1 [25] and increased expression of Scarb1 (**Fig. 4E**) [26], suggesting attenuated hepatic HDL production and accelerated hepatic HDL clearance, respectively. Although APOE*3-Leiden.CETP transgenic mice have a humanized lipoprotein metabolism, their BA pools obviously contain mouse/rat-specific MCAs. These hydrophilic BA species will have influenced some of the read-outs in the present study. In fact, we have recently shown that certain effects of FXR activation on cholesterol metabolism are blunted in mice with a more human-like BA pool composition [40]. For future studies on the interactions between BAs and lipoprotein metabolism, it may therefore be considered to generate mice that possess humanized lipoprotein metabolism in addition to a human-like BA pool composition.

In conclusion, the current study demonstrates that FXR agonism profoundly reduces adiposity and cures dyslipidemia in APOE*3-Leiden.CETP transgenic mice, a humanized mouse model of MetS. Although the results of the current study should be interpreted with caution given the species differences in BA metabolism between humans and mice, our data highlight the potential of pharmacological modulation of BA metabolism for the treatment of MetS-associated dyslipidemia.

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Author contributions

Y.P. designed the study, performed experiments, analyzed the data; E.Z., N.L.M., and M.K. contributed to animal experiments. J.C.W., K.W. P.C.N.R., J.A.K., C.K., Y.W., F.K., N.A.W. and A.K.G. interpreted data and revised the manuscript; J.F.B. designed the study and revised the manuscript constructively.

Conflict of interest

Dr. Claus Kremoser is CEO of Phenex Pharmaceuticals AG.

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Supplemental appendix

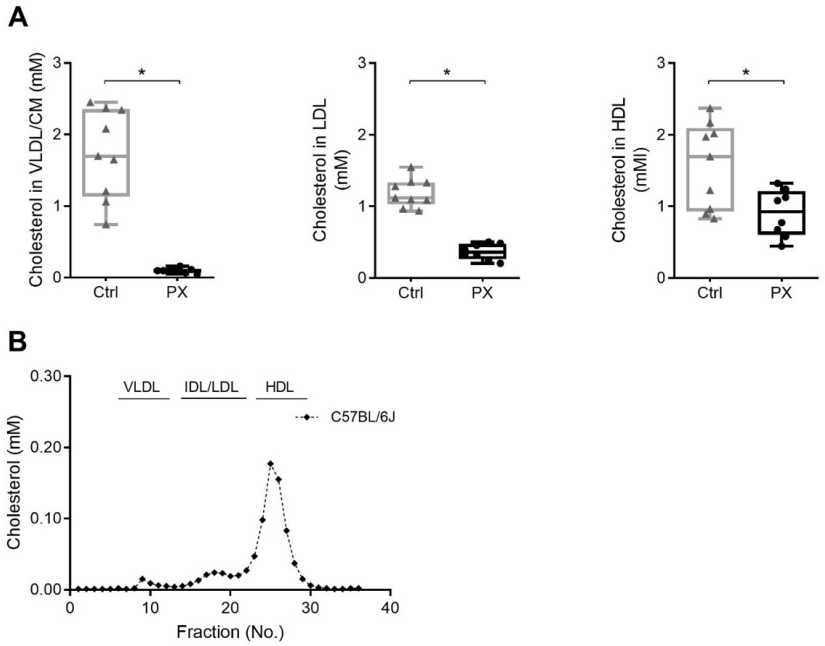

Figure S1. FXR stimulation profoundly decreases cholesterol contained within ApoB-containing lipoproteins. (A) Cholesterol content in lipoprotein fractions after 4 weeks of treatment with PX20606 (PX) in APOE*3-Leiden.CETP transgenic mice on Western-type diet. *Significant difference between groups, n=9 (Ctrl) and n=8 (PX) animals/group. (B) Cholesterol distribution over lipoprotein fractions after separation of pooled plasma samples (n=7) of young-adult male chow-fed C57BL/6J mice by FPLC. Ctrl, Control; PX, PX20606.

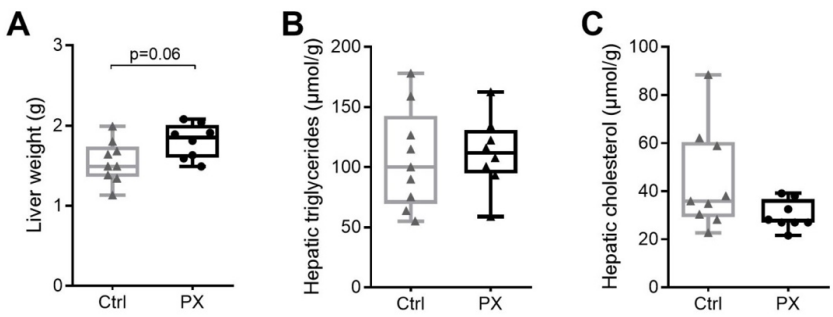

Figure S2. FXR stimulation does not impact liver lipid levels.

(A) Liver weight and (B) triglyceride and (C) cholesterol concentrations in livers from Western-type diet-fed APOE*3-Leiden.CETP transgenic mice treated with PX20606 (PX) for 4 weeks. n=9 (Ctrl) and n=8 (PX) animals/group. Ctrl, Control; PX, PX20606.