A fatty battle: towards identification of novel genetic targets to comBAT cardiometabolic diseases

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Chapter 4

The bile acid sequestrant colesevelam enhances the beneficial effects of brown adipose tissue activation on cholesterol metabolism in APOE*3-Leiden.CETP mice

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ABSTRACT

Background

We previously showed that brown adipose tissue (BAT) activation via β3-adrenergic receptor (AR) agonism increases the delivery of cholesterol to the liver via the uptake of cholesterol-enriched TRL remnants and HDL-cholesterol, thereby reducing atherosclerosis development. Liver cholesterol is the main substrate for the synthesis of bile acids (BAs) that are secreted into the intestine, from which the majority is reabsorbed into the portal circulation. Since the effect of BAT activation on hepatic cholesterol and BA metabolism are still obscure, we now aimed to evaluate the effects of BAT activation on cholesterol and BA metabolism without and with blocking the enterohepatic BA circulation.

Methods and Results

*APOE*3-Leiden.CETP* mice were fed a Western-type diet and treated without or with the selective β3-AR agonist CL316,243 to activate brown fat for 9 weeks. β3-AR agonism reduced fecal BA excretion, increased BA levels in plasma, and induced hepatic cholesterol accumulation. In a subsequent experiment, mice were fed a Western-type diet and received CL316,243 for 4 weeks without or with the BA sequestrant colesevelam that blocks BA reabsorption from the gut. Concomitant treatment with colesevelam increased fecal BA excretion, normalized plasma BA levels, and reversed the hepatic cholesterol accumulation caused by β3-AR agonism. Importantly, while β3-AR agonism alone reduced plasma cholesterol, concomitant treatment with colesevelam reduced plasma cholesterol even further.

Conclusions

BAT activation alone decreases plasma cholesterol while increasing plasma BA levels and hepatic cholesterol. BAT activation combined with BA sequestration improves BA metabolism, prevents the increase in hepatic cholesterol, and further reduces plasma cholesterol as compared to BAT activation alone. These data suggest that combining BAT activation with BA sequestration additively improves cholesterol metabolism and may further reduce atherosclerosis development.

INTRODUCTION

Brown adipose tissue (BAT) is a metabolically active organ present in *e.g.* rodents (1) and humans (2) and is responsible for nonshivering thermogenesis to defend body core temperature. Cold exposure, the most important physiological activator of BAT, leads to the release of noradrenalin from sympathetic nerves that innervate BAT. Noradrenalin subsequently binds to the β3-adrenergic receptor (β3-AR) on the membrane of the brown adipocytes, resulting in their activation to produce heat (3). Since heat combustion is an energy consuming process, activated BAT takes up large amounts of nutrients from the circulation, mainly triglyceride (TG)-derived fatty acids (FA) from TG-rich lipoproteins (TRLs; *i.e.* very-low density lipoproteins (VLDL) and chylomicrons) (4, 5). As a result, BAT activation accelerates the formation and hepatic uptake of cholesterol-enriched lipoprotein remnants, thereby protecting from hyperlipidemia and atherosclerosis development (4). This atheroprotective effect of BAT activation can be enhanced by concomitant treatment with a statin to accelerate remnant clearance (6). In addition to reducing cholesterol-enriched TRL remnant levels, β3-AR agonism also improves HDL functionality as reflected by increased reverse cholesterol transport (RCT) (7). Collectively, these studies thus show that β3-AR agonism increases cholesterol delivery towards the liver via both accelerating the clearance of cholesterol-enriched TRL remnants and improving HDL-mediated RCT (4, 6).

Hepatic cholesterol is the substrate for the synthesis of bile acids (BAs), which are important for the excretion of cholesterol (8), which can be synthesized via either the classical pathway or the alternative pathway. The classical pathway is controlled by the rate-limiting enzyme cholesterol 7-α-hydroxylase (CYP7A1) and results in the production of cholic acid (CA)-derived BAs. The alternative pathway is controlled by sterol 27-hydroxylase (CYP27A1) and 25-hydroxycholesterol 7 α-hydroxylase (CYP7B1), in which chenodeoxycholic acid (CDCA)-derived BA are synthesized. These pathways lead to the formation of primary BAs that are conjugated in the liver with taurine or glycine and transported towards the gall bladder from which they are transported to the intestines (9). Most of the conjugated primary BAs are actively reabsorbed in the terminal part of the ileum, mainly via the transporter apical sodium dependent BA transporter (ASBT). Alternatively, they can be modified by the gut microbiota into secondary BAs, followed by either reabsorption via passive diffusion in the colon or excretion from the body via the feces (10). The reabsorbed BAs are transported towards the liver where they

are taken up, mainly via sodium taurocholate cotransporting polypeptide (NTCP) (8). Of note, derailment of BA metabolism can also cause accumulation of cholesterol in the liver.

Since β3-AR agonism increases cholesterol delivery towards the liver (6), and BA metabolism largely orchestrates the fate of hepatic cholesterol, the aim of the current study was to evaluate the effects of β3-AR agonism on hepatic cholesterol and BA metabolism. In addition, we assessed whether intestinal BA sequestration influences the effects of β3-AR agonism on cholesterol and BA metabolism. To this end, we first treated *APOE*3-Leiden.CETP* (*E3L.CETP*) mice, a well-established model of human-like lipoprotein metabolism (11-13), with the selective β3-AR agonist CL316,243 to activate BAT and assessed cholesterol and BA metabolism. Next, we treated *E3L.CETP* mice with vehicle, CL316,243, or/and the BA sequestrant colesevelam to block reabsorption of BAs.

MATERIALS AND METHODS

Animals and treatment

Hemizygous *APOE*3-Leiden* (*E3L*) mice were crossbred with homozygous human cholesteryl ester transfer protein (CETP) transgenic mice to generate heterozygous *E3L.CETP* mice (14). In all studies described below, 10-12 weeks old female *E3L.CETP* mice were housed under standard conditions in conventional cages with a 12-hours light/dark cycle, at room temperature (22°C) and with *ad libitum* access to food and water. During all studies mice were fed a Western-type diet (WTD; AB diets, Woerden, The Netherlands) containing 15% cacao butter, 1% corn oil and 0.15% (w/w) cholesterol.

In a first experiment, mice were randomized into 2 groups (based on plasma lipid levels and fat mass) after a run-in period of 6 weeks and treated 5 days/week with vehicle (PBS; symbol: -) or the β3-AR agonist CL316,243 (symbol: β; Tocris Bioscience Bristol, United Kingdom; 20 μg/mouse) by subcutaneous injections between 14.00 and 16.00 h for an additional 9 weeks.

In the second experiment, mice were randomized into 2 groups (based on plasma lipid levels and fat mass) after a run-in period of 3 weeks in which they received WTD supplemented without or with colesevelam (symbol: c; 0.15%, w/w). After an additional

run-in period of 3 weeks, mice in each treatment group were randomized into 2 subgroups (based on plasma lipid levels and fat mass) and additionally treated 5 days/ week with vehicle or the selective β3-AR agonist CL316,243 for an additional 4 weeks. This resulted in the following 4 treatment groups: 1) vehicle (-), 2) CL316,243 (β), 3) colesevelam (c), and 4) colesevelam + CL316,243 (c+β).

Food intake was monitored 3 times a week. Body weight and body composition (body fat and lean mass; EchoMRI-100; EchoMRI, Houston, TX, USA) were monitored weekly. All animal experiments were performed in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and have received approval from the Animal Ethical Committee (Leiden University Medical Center, Leiden, The Netherlands).

Plasma parameters

Blood was collected from the tail vein of 4 h fasted mice into capillaries that were subsequently placed on ice and centrifuged, and plasma was assayed for TG and total cholesterol (TC) using enzymatic kits from Roche Diagnostics (Mannheim, Germany). To measure HDL-C levels, apoB-containing lipoproteins were precipitated from plasma with 20% polyethylene glycol 6,000 in 200 mmol/L glycine buffer (pH 10), and cholesterol was measured in the supernatant as described above. Plasma nonHDL-C levels were calculated by subtraction of HDL-C from TC levels.

Bile acid analysis

Bile acid composition was determined in feces and plasma. Feces of animals (first experiment: 2 mice/cage, after 8 weeks of treatment; second experiment: individually housed animals, after 4 weeks of treatment) was collected over a 24-hour period. Fecal samples were dried at room temperature, weighed and homogenized. Bile acid and neutral sterol composition were determined in an aliquot of feces and in 5 µL bile by gas liquid chromatography (GC) as described previously (15-17) and modified as follows. Fecal BAs were solubilized from 50 mg feces in methanol/0.1 M sodium hydroxide (3:1) at 80°C for 2 h. As an internal standard 15 nmol of 5β-cholanic acid 7α,12α diol was added. Next, BAs were extracted using C-18 columns and eluted with 75% methanol followed evaporation at 65°C under a stream of nitrogen. Subsequently, BAs were deconjugated enzymatically by adding choloyl glycine hydrolase at 37°C for 15 h in sodium acetate buffer pH 5.6. Next, BAs were extracted again using C-18 columns as described above. After drying the samples under nitrogen the BAs

were methylated using acetylchloride/methanol at 55°C. Subsequently, a trimethylsilyl-derivative was made with N_2O-bis/trimethylsilyltrifluoroacetuylamide, pyridine and trimethylsilane. BA were diluted in hexane and analyzed by GC (Agilent 6890, Amstelveen, the Netherlands) using a CPSil 19 capilliary column (25 m x 0.25 mm x 0.2 µm) (Chrompack, Middelburg, The Netherlands). The total amount of BAs or neutral sterols was calculated as the sum of the individually quantified bile acids or neutral sterols.

BA concentration in plasma was measured in 25 µL of homogenized plasma (first experiment: after 9 weeks of treatment; second experiment: after 4 weeks of treatment). An internal standard containing D4-cholate, D4-chenodeoxycholate, D4-glycocholate, D4-taurocholate, D4-glycochenodeoxycholate and D4-taurochenodeoxycholate was added. Subsequently, samples were mixed and centrifuged at 15,900 g. The supernatant was removed, evaporated under vacuum at 40°C and reconstituted in 100 µL of 50% methanol. Bile acid profile was measured using liquid chromatography tandem MS (LC-MS/MS) as described (18).

Biliary bile acid secretion

In the second experiment, after 4 weeks of treatment, mice were anesthetized with Hypnorm (fentanyl/fluanisone; 1 mL/kg) and diazepam (10 mg/kg) and gallbladder cannulation was performed to collect hepatic bile. After a 5 min equilibration period, bile was collected for 15 min while mice were laying on heating matrasses to maintain body temperature.

Hepatic lipid composition

At the end of both studies, mice were killed by cervical dislocation. In experiment 2, this was done after 15 min of bile collection. Blood was drawn by cardiac puncture for serum isolation. Subsequently, mice were perfused with ice-cold PBS and organs and tissues were weighed and collected for further analysis. Pieces of liver were collected to extract liver lipids as previously described (11). In brief, small liver pieces (approx. 50 mg) were homogenized in ice-cold methanol (10 µL/mg tissue). Lipids were extracted into an organic phase by the addition of 1,800 µL CH_3OH : $CHCl_3$ (1:3, v/v) to 45 µL homogenate. After vigorous vortexing and phase separation by centrifugation (14,000 rpm; 15 min at room temperature), the organic phase was dried and dissolved in 2% Triton X-100. TG, TC (both as described above) and PL concentrations (Instruchemie, Delfzijl, The Netherlands) were measured using commercial kits. Liver lipids were expressed as nmol/mg protein as determined using the BCA protein assay kit (Pierce, Rockford, IL, USA).

Gene expression analysis

RNA was extracted from snap-frozen tissues (approx. 25 mg) using Tripure RNA Isolation reagent (Roche) according to the manufacturer's protocol. Total RNA (1 µg) was reverse transcribed using Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Promega) for RT-qPCR according to the manufacturer's instructions to produce cDNA. mRNA expression was normalized to β*2-microglobulin* mRNA expression and expressed as fold change compared to vehicle-treated mice using the ΔΔCT method. The primer sequences used are listed in Table 1 below.

Table 1: Primer sequences[1]

Gene	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')
Abcg5	TGTCCTACAGCGTCAGCAACC	GGCCACTCTCGATGTACAAGG
β2-microglobulin	TGACCGGCTTGTATGCTATC	CAGTGTGAGCCAGGATATAG
Bsep	CTGCCAAGGATGCTAATGCA	CGATGGCTACCCTTTGCTTCT
Cd3	AACACGTACTTGTACCTGAAAGCTC	GATGATTATGGCTACTGCTGTCA
Cd4	ACACACCTGTGCAAGAAGCA	GCTCTTGTTGGTTGGGAATC
Cd8	GGCTCTGGCTGGTCTTCA	GACGAAGGGGTCTGAATGAG
Cd68	ATCCCCACCTGTCTCTCTCA	TTGCATTTCCACAGCAGAAG
Cyp7a1	CAGGGAGATGCTCTGTGTTCA	AGGCATACATCCCTTCCGTGA
Cyp7b1	CAGCTATGTTCTGGGCAATG	TCGGATGATGCTGGAGTATG
Cyp8b1	GGACAGCCTATCCTTGGTGA	CGGAACTTCCTGAACAGCTC
Cyp27a1	TCTGGCTACCTGCACTTCCT	CTGGATCTCTGGGCTCTTTG
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
Fxr	GGCCTCTGGGTACCACTACA	ACATCCCCATCTCTTTGCAC
Gapdh	GGGGCTGGCATTGCTCTCAA	TTGCTCAGTGTCCTTGCTGGGG
Hsd3b7	CCATCCACAAAGTCAACGTG	CTCCATTGACCTTCCTTCCA
Il-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
Mcp-1	GCATCTGCCCTAAGGTCTTCA	TTCACTGTCACACTGGTCACTCCTA
Ntcp	CTTCACTGCCTTGGGCATGATG	TGTTTCCATGCTGATGGTGCG
Oatp1a1	TGAGAAAGACAGCAGTAGGACTTT	GTGATTTGGCTAGGTATGCAC
Ost-α	TGGACCCTGGAAGACATA	TAACCACTGATAAGGCTGAG
Ost-β	GACCACAGTGCAGAGAAAGC	ATTCCAAGGAGCCGCATCT
Tnfa	AGCCCACGTCGTAGCAAACCAC	TCGGGGCAGCCTTGTCCCTT

[1]*Abcg5*, ATP-binding cassette sub-family G member 5; *Bsep*, bile salt export pump; *Cyp7a1*,cholesterol 7 alpha-hydroxylase; *Cyp7b1*, oxysterol and steroid 7-alpha-hydroxylase; *Cyp8b1*, sterol 12-alpha-hydroxylase; *Cyp27a1*, sterol 27-hydroxylase; *Fxr*, farnesoid X receptor; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Hsd3b7*, hydroxy-delta-5-steroid dehydrogenase; *Il-1β*, interleukin 1β; *Mcp-1*, monocyte chemoattractant protein-1; *Ntcp*, Na^+ taurocholate cotransporting polypeptide; *Oatp1a1*, organic anion transporter family member 1α1; *Ost-α*, organic solute transporter α; *Ost-β*, organic solute transporterβ; *Tnfa*, tumor necrosis factor-α.

Histology

During necropsy, livers were removed and samples were fixed in 4% paraformaldehyde, dehydrated in 70% EtOH and embedded in paraffin. Tissue sections (5 µm) were stained with hematoxylin and eosin (HE) using standard protocols. Hepatic inflammation was determined by counting the number of inflammatory foci per mm^2 liver section. The accumulation of more than 5 macrophages/monocytes is defined as one inflammatory focus. To determine the number of F4/80-positive cells per mm^2, paraffin-embedded liver sections were sectioned using Leica CM3050 S cryostat. After deparaffinization and rehydration, the liver sections were blocked with 2.5% goat serum blocking solution (VECTOR). Liver sections were incubated overnight with rat anti-murine F4/80 antibody (AbD Serotec) followed by a goat anti-rat IgG/HRP (VECTOR) secondary antibody. Tissue sections were then developed by Nova RED (Vector SK-4800) and counterstained with Mayers Hematoxylin (Merck). The final products were mounted with Histomount/Xylene (National Diagnostics) on glass slides. Subsequently, the glass slides were scanned in the Philips Ultra Fast Scanner 1.6 RA (Philips) and the results were processed and viewed on the Philips IMS (Image Management System) Web Viewer.

Statistical analysis

In the first study, differences between vehicle and CL316,243 treated mice were determined using Student T-Test. For the second study, differences between groups were determined using one-way ANOVA with the LSD posthoc test. Probability values less than 0.05 were considered statistically significant. Data are presented as mean ± SEM. All statistical analyses were performed with the SPSS 20.0 software package for Windows (SPSS, Chicago, IL USA).

RESULTS

β3-AR agonism increases hepatic cholesterol accumulation

We previously observed that β3-AR agonism increases TG-derived FA uptake by BAT and consequently accelerates hepatic uptake of cholesterol-enriched TRL remnants (6) as well as reverse cholesterol transport to the liver (7). To evaluate the effect of the increased hepatic cholesterol delivery on hepatic cholesterol levels, *E3L.CETP* mice were treated with the β3-AR agonist CL316,243 (β) compared to vehicle (-) for 9 weeks. β3-AR agonism clearly increased hepatic cholesterol levels (+30%, $p<0.05$; **Fig. 1A**). In

addition, histological examination showed that β3-AR agonism increased the number of inflammatory foci (+106%, p<0.05, **Fig. 1B**) and tended to increase the number of F4/80-positive cells (+27%, p<0.12; **Fig. 1C**), without influencing the mRNA expression of the macrophage markers *F4/80* and *Cd68* (**Fig. 1D**) or the pro-inflammatory cytokines tumour necrosis factor α (*Tnfα*), interleukin-1β (*Il-1β*) and monocyte chemotactic protein 1 (*Mcp1*) (**Fig. 1E**). However, β3-AR agonism significantly increased mRNA expression of the T-cell markers *Cd3* (+37%, p<0.05) and tended to increase *Cd4* (+36%, p=0.06) (**Fig. 1F**). Collectively, these data show that long-term β3-AR agonism increases hepatic cholesterol as well as inflammation.

β3-AR agonism reduces the expression of genes involved in bile acid production

Since hepatic cholesterol accumulation may result from disturbances in BA metabolism, we measured mRNA expression of genes involved in hepatic BA metabolism. β3-AR agonism non-significantly reduced *Cyp7a1* (-28%, n.s.), and significantly reduced *Hsd3b7* (-57%, p<0.01) and *Cyp8b1* (-40%, p<0.01; **Fig. 2A**), which are all involved in the classical BA synthesis pathway. In addition, β3-AR agonism reduced *Cyp27a1* (-53%, p<0.01) and *Cyp7b1* (-38%, p<0.001; **Fig. 2B**), which are involved in the alternative BA synthesis pathway. β3-AR agonism also tended to reduce *Abcg5* (-31%, p=0.06) and *Bsep* (-27%, p=0.05; **Fig. 2C**), involved in excretion of sterols and BAs, respectively, towards the gall bladder. β3-AR agonism also increased expression of *Ost-β* (+64%, p<0.01; **Fig. 2D**), involved in the basolatoral BA secretion from the liver towards the systemic circulation. β3-AR agonism tended to reduce mRNA expression of both *Oatp1a1* (-60%, p=0.06) and *Ntcp* (-37%, p=0.08; **Fig. 2E**), involved in the uptake of reabsorbed BAs by the liver. Long-term β3-AR agonism thus reduces the expression of genes that are involved in BA production.

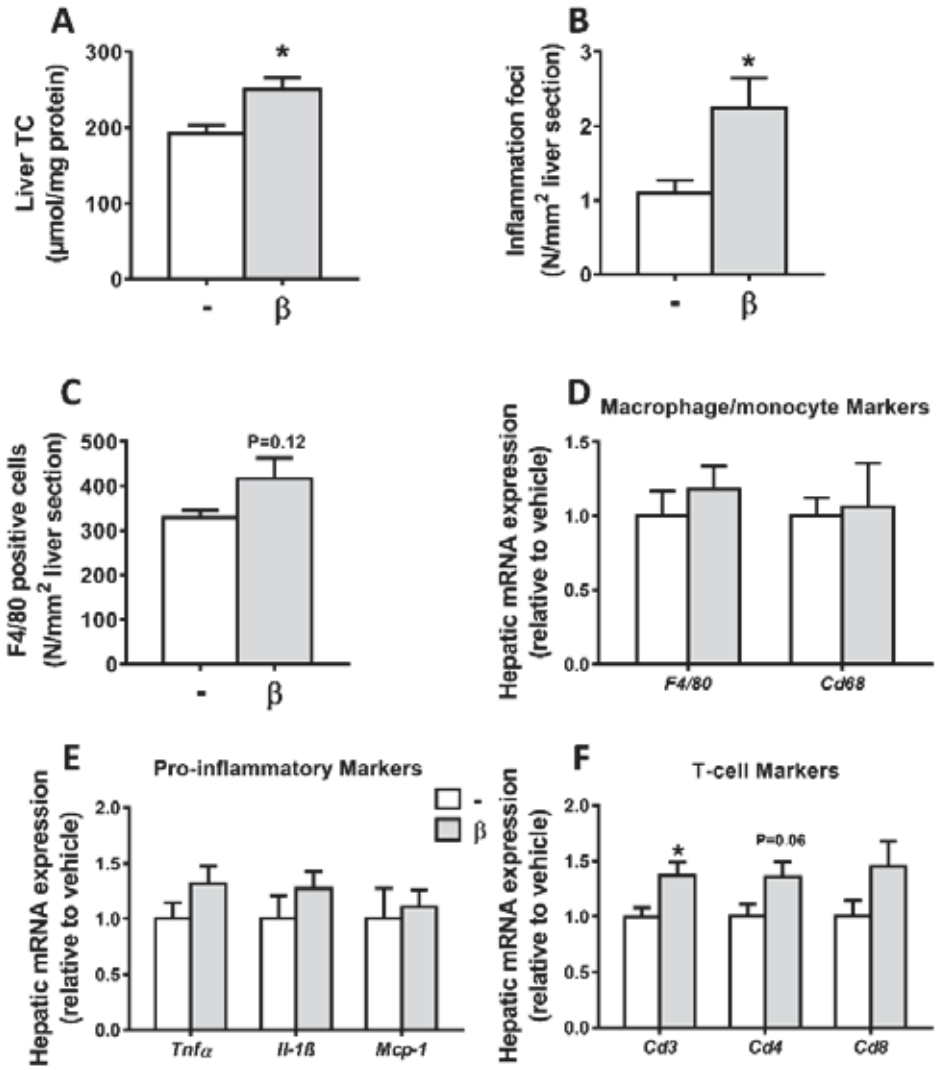

Figure 1: *The effect of β3-AR agonism on hepatic inflammation parameters. Female E3L.CETP mice on a Western-type diet supplemented with 0.15% cholesterol were treated for 9 weeks with vehicle (-) or β3-AR agonism (β). After 9 weeks of treatment, liver samples were collected and (A) liver total cholesterol levels were measured. Next, livers were histologically examined and the number of (B) inflammatory foci and (C) F4/80 positive cells was determined. RT-qPCR was used to measure mRNA expression of (D) macrophage and monocyte markers, (E) pro-inflammatory markers and (F) T-cell markers (n=8/group). Values are means ± SEM. *p<0.05.*

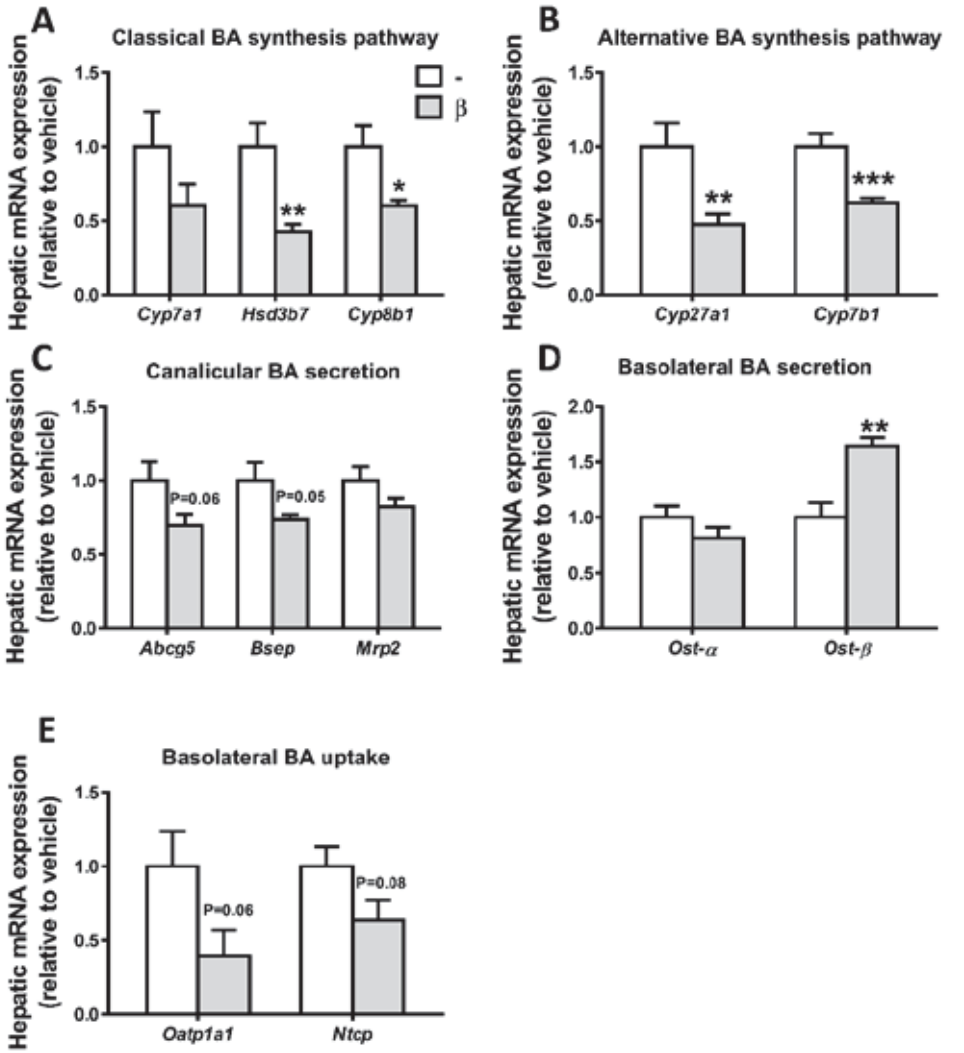

Figure 2: *The effect of β3-AR agonism on expression of genes involved in bile acid metabolism. Female E3L.CETP mice on a Western-type diet supplemented with 0.15% cholesterol were treated for 9 weeks with vehicle (-) or β3-AR agonism (β). After 9 weeks of treatment, liver samples were collected and RT-qPCR was used to measure mRNA expression of genes involved in (A) the classical bile acid (BA) synthesis pathway, (B) the alternative BA synthesis pathway (C) canicular BA secretion (D) basolateral BA secretion and (E) basolateral BA uptake (n=8/group). Values are means ± SEM. *p<0.05; **p<0.01; ***p<0.001.*

β3-AR agonism decreases fecal bile acid excretion and increases plasma bile acid levels

To evaluate whether the changes in mRNA expression of genes involved in BA metabolism related to changed BA concentrations, fecal and plasma BAs were determined after 8-9 weeks of treatment. β3-AR agonism reduced the total BA content in feces (-32%, p<0.01; **Fig. 3A**), without influencing total fecal output (data not shown). While CA-derived BAs (*i.e.* derived from the classical pathway) were non-significantly lowered (-27%, p=0.12; **Fig. 3B**), CDCA-derived BAs (*i.e.* derived from the alternative pathway) were clearly reduced (-36%, p<0.05; **Fig. 3C**). In contrast, plasma levels of total BAs were largely increased after β3-AR agonism (+198%, p<0.001; **Fig. 3D**) and this was due to an increase in both CA-derived BAs (+187%, p<0.001; **Fig. 3E**) and CDCA-derived BAs (+117%, p<0.001; **Fig. 3F**). The reduction in fecal BAs accompanied by an increase in plasma BAs suggest that long-term β3-AR agonism increases BA reabsorption by the gut.

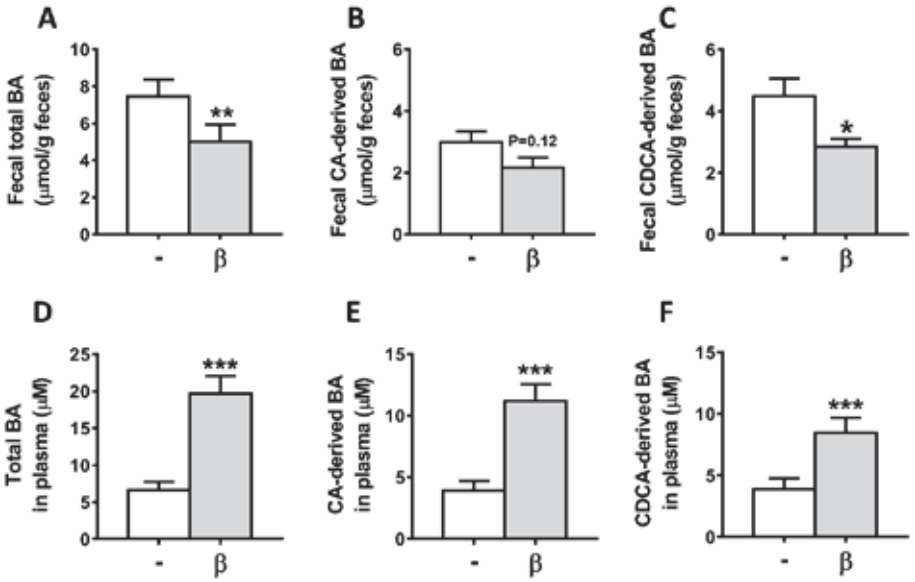

Figure 3: *The effect of β3-AR agonism on fecal and plasma BA levels. Female E3L.CETP mice on a Western-type diet supplemented with 0.15% cholesterol were treated for 9 weeks with vehicle (-) or β3-AR agonism (β). After 8 weeks of treatment, feces was collected and used to measure (A) total bile acids (BA), (B) CA-derived BA and (C) CDCA-derived BA (n=7-8/group). After 9 weeks of treatment, plasma samples were collected and used to measure (D) total BA, (E) CA-derived BA and (F) CDCA-derived BA. (n=14-16/group) Values are means ± SEM. *p<0.05; **p<0.01; ***p<0.001.*

Colesevelam on top of β3-AR agonism additively improves plasma cholesterol levels

Long-term β3-AR agonism thus seems to increase reabsorption of BAs in the intestines that may increase hepatic cholesterol by downregulation of hepatic BA synthesis. We therefore hypothesized that inhibition of BA reabsorption would protect against hepatic cholesterol accumulation. To test this hypothesis, mice were treated for 4 weeks with vehicle, β3-AR agonism alone, colesevelam alone, which inhibits reabsorption of BAs by sequestration in the intestine, or the combination of colesevelam and β3-AR agonism.

None of the treatments influenced food intake (**Fig. 4A**), body weight (**Fig. 4B**) or lean mass (**Fig. 4C**). While β3-AR agonism alone tended to reduce fat mass (-20%, p=0.09), colesevelam had no effect, and colesevelam on top of β3-AR agonism reduced fat mass as compared to vehicle and colesevelam alone (both approx. -40%, p<0.01; **Fig. 4D**). In line with these effects on fat mass, β3-AR agonism alone reduced gonadal white adipose tissue (gWAT) weight (-36%, p<0.01) as compared to vehicle. Colesevelam alone did not influence gWAT weight, but the addition of colesevelam on top of β3-AR agonism reduced gWAT weight with as compared to vehicle (-47%, p<0.001) and colesevelam alone (-49%, p<0.01; **Fig. 4E**). Liver weight was not significantly influenced by β3-AR agonism alone or in combination with colesevelam (**Fig. 4E**).

Next, we assessed the effect of colesevelam on top of β3-AR agonism on plasma lipid levels. After 4 weeks of treatment, plasma TG levels were reduced by β3-AR agonism alone (-52%, p<0.01) and tended to be reduced by colesevelam alone (-33%, p=0.07) as compared to vehicle. Colesevelam on top of β3-AR agonism reduced plasma TG levels as compared to vehicle (-74%, p<0.001), and also as compared to colesevelam alone (-62%, p<0.05; **Fig. 5A**). In addition, colesevelam alone reduced plasma TC levels as compared to vehicle (-47%, p<0.001). Colesevelam on top of β3-AR agonism also reduced plasma TC levels as compared to vehicle (-55%, p<0.001) and β3-AR agonism alone (-49%, p<0.001; **Fig. 5B**).

Since cholesterol can be carried by either pro- or anti-atherogenic lipoprotein classes, we next determined the distribution of cholesterol over plasma non-HDL and HDL. Plasma non-HDL-C levels tended to be reduced by β3-AR agonism alone (-27%, p=0.05), and were significantly reduced by colesevelam alone (-55%, p<0.001) and colesevelam on top of β3-AR agonism (-68%, p<0.001) as compared to vehicle. Moreover, colesevelam

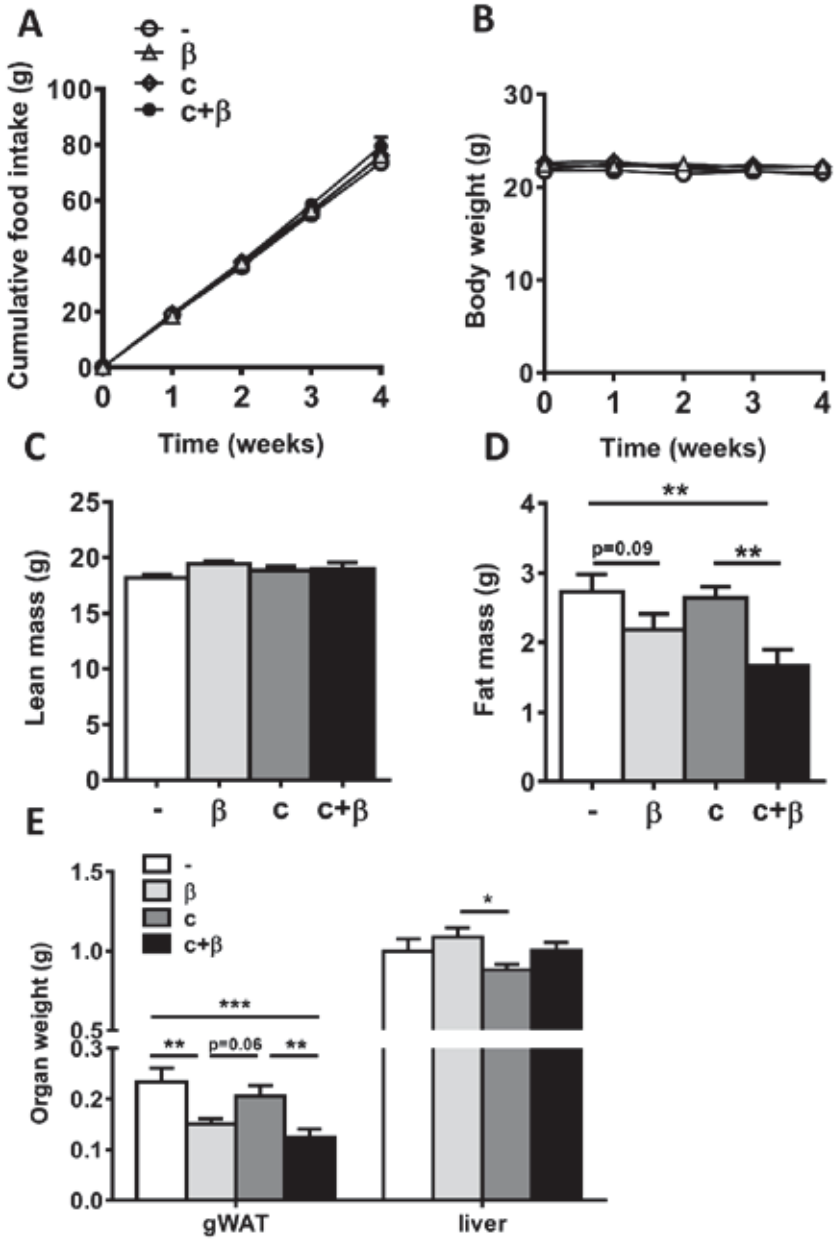

Figure 4: *The effect of β3-AR agonism, colesevelam and colesevelam combined with β3-AR agonism on metabolic characteristics. Female E3L.CETP mice on a Western-type diet supplemented with 0.15% cholesterol were treated for 9 weeks with vehicle (-), β3-AR agonism (β), colesevelam (c), or the combination (c+β) (n=9/group). At the indicated time points, (A) cumulative food intake and (B) body weight were measured. At the end of the intervention, (C) body lean mass and (D) body fat mass were determined. Mice were killed and organs were collected and (E) weighed. Values are means ± SEM. *p<0.05; **p<0.01; ***p<0.001.*

on top of β3-AR agonism further reduced non-HDL-C levels as compared to β3-AR agonism alone (-56%, p<0.001; **Fig. 5C**). In addition, both β3-AR agonism alone (+34%, p<0.05), and in combination with colesevelam (+52%, p<0.01; **Fig. 5D**) increased anti-atherogenic HDL-C levels as compared to vehicle. These findings indicate that colesevelam does not enhance the TG-lowering effect and HDL-C-increasing effect of β3-AR agonism alone, but does additively reduce plasma non-HDL-C levels.

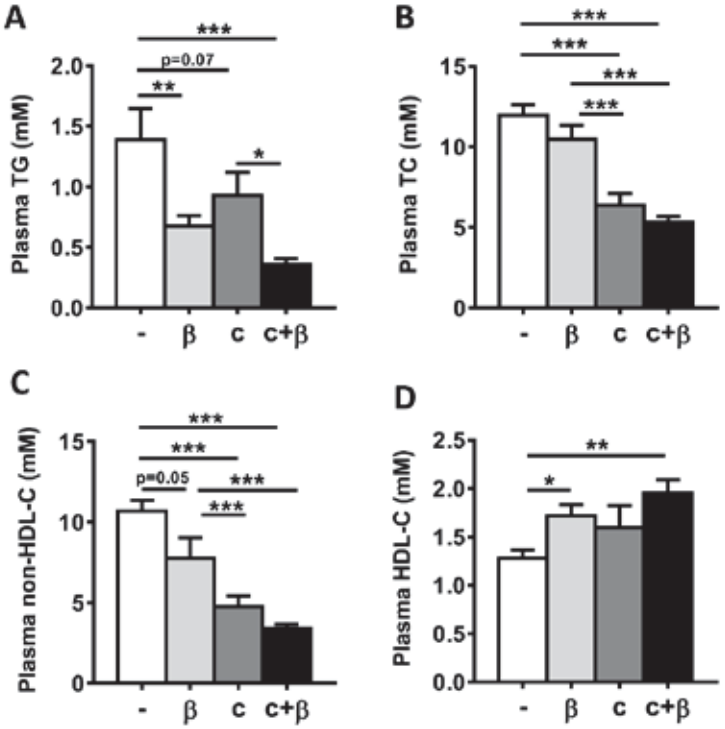

Figure 5: *The effect of β3-AR agonism, colesevelam and colesevelam combined with β3-AR agonism on plasma lipid levels. Female E3L.CETP mice on a Western-type diet supplemented with 0.15% cholesterol were treated with vehicle (-), β3-AR agonism (β), colesevelam (c) or the combination (c+β). After 4 weeks of treatment, 4-h fasting plasma lipid levels of (A) TG, (B) TC, (C) nonHDL-cholesterol and (D) HDL-cholesterol were measured (n=9/group). Values are means ± SEM. *p<0.05; **p<0.01; ***p<0.001.*

Colesevelam on top of β3-AR agonism reverses hepatic cholesterol accumulation

We next evaluated whether the addition of colesevelam could reverse hepatic cholesterol accumulation as induced by β3-AR agonism. Colesevelam alone reduced hepatic TC levels as compared to vehicle (-41%, p<0.01) More importantly, colesevelam on top of β3-AR agonism also largely reduced hepatic TC levels as compared to vehicle (-45%, p<0.001) and β3-AR

agonism alone (-53%, p<0.001; **Fig. 6A**), and to similar levels as colesevelam alone. While hepatic TG levels were not influenced by any of the treatments (**Fig. 6B**), colesevelam on top of β3-AR agonism tended to lower hepatic phospholipid levels as compared to vehicle (-12%, p=0.06) and β3-AR agonism alone (-12%, p=0.07; **Fig. 6C**).

In line with our previous observation (**Fig. 1B**), β3-AR agonism caused hepatic inflammation as indicated by an increased number of inflammatory foci in the liver (+107%, p<0.05). Colesevelam on top of β3-AR agonism also increased the number of inflammatory foci as compared to vehicle (+89%, p<0.05; **Fig. 6D**). The combination of colesevelam and β3-AR agonism reduced mRNA expression of *F4/80* as compared vehicle (-37%, p<0.01), β3-AR agonism (-26%, p=0.07) and colesevelam (-35%, p<0.01; **Fig. 6E**). The mRNA expression of *Mcp-1* was increased by colesevelam as compared to vehicle (+24%, p<0.05; **Fig. 6F**), while the other treatments did not influence its expression. Colesevelam on top of β3-AR agonism thus strongly reduces the elevated hepatic cholesterol levels as induced by β3-AR agonism, but does not influence hepatic inflammation.

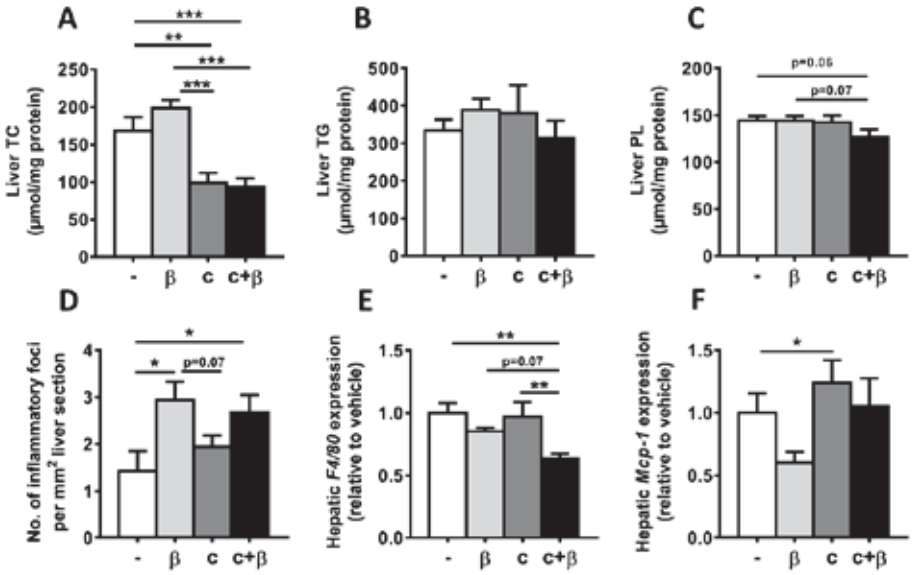

Figure 6: *The effect of β3-AR agonism, colesevelam and colesevelam combined with β3-AR agonism on liver lipids and inflammation. Female E3L.CETP mice on a Western-type diet supplemented with 0.15% cholesterol were treated with vehicle (-), β3-AR agonism (β), colesevelam (c), or the combination (c+β). After 4 weeks of treatment, liver samples were collected lipids were extracted from liver samples, and (A) TC, (B) TG and (C) phospholipid (PL) content was determined (n=9/group). The number of (D) inflammatory foci was determined in liver slices. qPCR was used to measure mRNA expression of (E) F4/80 and (F) Mcp1 (n=8/group). Values are means ± SEM. *p<0.05; **p<0.01; ***p<0.001.*

Colesevelam on top of β3-AR agonism largely increases fecal bile acid excretion

We next evaluated the effect of the treatments on the expression of genes involved in BA metabolism. Hepatic mRNA expression of *Cyp7a1* was reduced by all treatments as compared to vehicle, which reached significance for colesevelam alone only (-43%, $p<0.05$; **Fig. 7A**). Expression of *Cyp7b1* was increased by β3-AR agonism alone (+33%, $p<0.05$) and tended to be increased by colesevelam alone (+24%, $p=0.08$; **Fig. 7B**) as compared to vehicle. Expression of hepatic *Abcg5* and expression of *Asbt* in ileum were similar between all groups (**Fig. 7C, D**), but *Ost-α* was largely increased by colesevelam as compared to the other groups (approx. +50%, $p<0.05$; **Fig. 7E**). These data show that colesevelam on top of β3-AR agonism does not influence the expression of genes involved in BA metabolism.

We next determined the biliary cholesterol excretion by performing bile cannulation. As compared to vehicle, the bile flow was increased by β3-AR agonism alone (+47%, $p<0.01$), by colesevelam alone (+26%, $p<0.05$) and by colesevelam on top of β3-AR agonism (+34%, $p<0.05$; **Fig. 7F**). Based on the bile flow we first calculated the BA production rate, which was increased in all treatments and reached significance for β3-AR agonism alone (+45, $p<0.05$; **Fig. 7G**). Next biliary cholesterol excretion was calculated, which was increased by β3-AR agonism (+47%, $p<0.05$; **Fig. 7H**), not influenced by colesevelam alone, and non-significantly increased by colesevelam on top of β3-AR agonism as compared to vehicle. Fecal neutral sterol excretion was not different between the groups (**Fig. 7I**). Finally, we measured fecal and plasma BAs. Total fecal BA excretion was reduced by β3-AR agonism alone (-46%, $p<0.05$) and non-significantly increased by colesevelam (+31%, n.s.). Colesevelam on top of β3-AR agonism increased fecal BA excretion as compared to vehicle (+70%, $p<0.05$) and β3-AR agonism alone (+212%, $p<0.001$; **Fig. 8A**). A similar pattern was observed for the effect of β3-AR agonism alone and in combination with colesevelam on fecal CA-derived BA excretion. In addition, colesevelam significantly increased fecal CA-derived BA excretion as compared to vehicle (+98%, $p<0.01$; **Fig. 8B**). Fecal CDCA-derived BA excretion was reduced by β3-AR agonism alone (-53%, $p<0.01$), while colesevelam on top of β3-AR agonism increased fecal CDCA-derived BA excretion as compared to β3-AR agonism alone (+99%, $p<0.05$; **Fig. 8C**), but not as compared to vehicle. Total plasma BAs were non-significantly increased by β3-AR agonism (+45%, n.s.; **Fig. 8D**). β3-AR agonism increased plasma CA-derived BAs (+59%, $p<0.05$; **Fig. 8E**), and non-significantly increased CDCA-derived BAs (+32%, n.s.) (**Fig. 8F**). These effects of β3-AR agonism were reversed by colesevelam (**Fig. 8E, F**), albeit that differences are not significant probably related to lack of power.

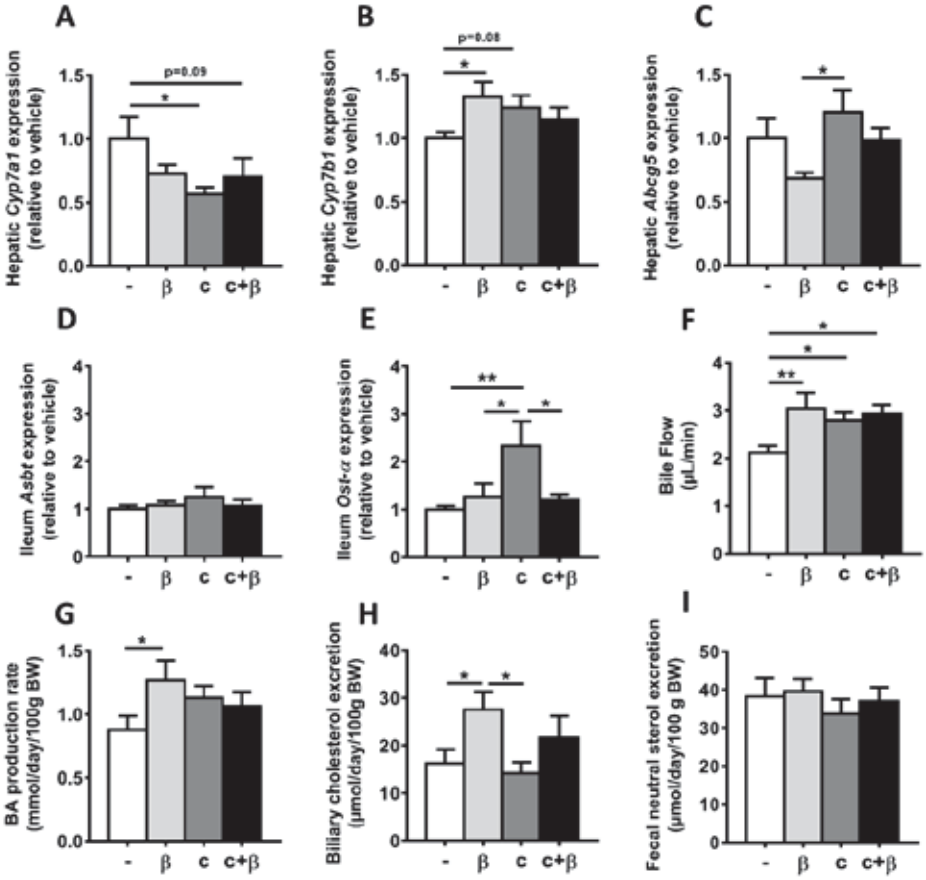

Figure 7: *The effect of β3-AR agonism, colesevelam and colesevelam combined with β3-AR agonism on expression of genes involved in bile acid metabolism. Female E3L.CETP mice on a Western-type diet supplemented with 0.15% cholesterol were treated with vehicle (-), β3-AR agonism (β), colesevelam (c), or the combination (c+β). After 4 weeks of treatment, livers were collected to measure mRNA expression of (A) Cyp7a1, (B) Cyp7b1, (C) Abcg5. Ileum was also collected to measure mRNA expression of (D) Asbt and (E) Ost-α. Gall bladders of mice were cannulated and (F) bile flow was determined and used to calculate (G) bile acid production rate and (H) biliary cholesterol excretion. Feces was collected to determine the (I) neutral sterol excretion (n=8/group). Values are means ± SEM. *p<0.05; **p<0.01; ***p<0.001.*

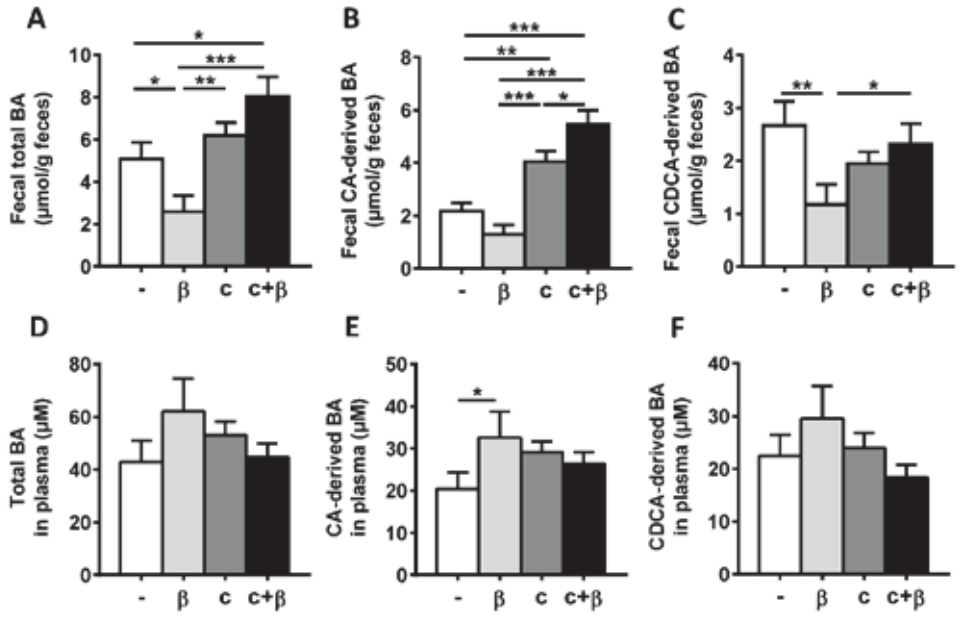

Figure 8: *The effect of β3-AR agonism, colesevelam and colesevelam combined with β3-AR agonism on expression of genes involved in bile acid metabolism. Female E3L.CETP mice on a Western-type diet supplemented with 0.15% cholesterol were treated with vehicle (-), β3-AR agonism (β), colesevelam (c), or the combination (c+β). After 4 weeks of treatment, fecal and plasma samples were collected and used to measure (A, D) total BA, (B, E) CA-derived BA and (C, F) CDCA-derived BA. (n=8/group) Values are means ± SEM. *p<0.05.*

DISCUSSION

Activated BAT is an important contributor to lipid metabolism by reducing hyperlipidemia and increasing HDL functionality, both of which contribute to the atheroprotective effects of BAT activation. The aim of the current study was to evaluate the effects of β3-AR agonism on hepatic cholesterol and BA metabolism, and the effects of BA sequestration thereon. Our data demonstrate that prolonged β3-AR reduces fecal BA secretion and increases BA levels in plasma, accompanied by increased hepatic cholesterol levels. In addition, we show that the BA sequestrant colesevelam on top of β3-AR agonism increases fecal BA excretion, normalizes plasma BA levels, and lowers cholesterol levels in liver and plasma as compared to β3-AR agonism alone.

We previously observed that prolonged β3-AR agonism in mice increases the delivery

of cholesterol to the liver via the uptake of cholesterol-enriched TRL remnants (6) and HDL-cholesterol (7). In line with the notion that hepatic cholesterol is the main substrate for the production of BAs, BAT activation by means of short-term cold exposure (*i.e.* 1 week) increases BA production rate and fecal BA excretion in mice (19). In the current study, we also observed that 4 weeks of treatment with a β3-AR agonist increases the bile acid production rate. However, fecal BA excretion was reduced while plasma BA levels were increased, and these effects were even more pronounced after 9 weeks of β3-AR agonism. High plasma BA levels are often accompanied by high levels of hepatic BA that reduce BA synthesis by a feed-back mechanism. Indeed, β3-AR agonism decreased mRNA expression of genes involved in the classical and alternative BA production. More importantly, β3-AR agonism increased hepatic cholesterol levels, likely as a result of increased cholesterol delivery to the liver (6, 7) in combination with decreased hepatic cholesterol utilization due to reduced BA synthesis. These results combined indicate that β3-AR agonism initially increases BA production and fecal BA excretion, while prolonged treatment with a β3-AR agonist increases the reabsorption of BAs by the gut, leading to increased plasma levels of BAs that are taken up by the liver to downregulate BA synthesis and thereby induce hepatic cholesterol accumulation.

Colesevelam is a BA sequestrant that reduces the reabsorption of BAs, mainly of deoxycholic acid (DCA; *i.e.* the main CA-derived BA) from the gut (8). Likely via this mechanism, colesevelam increased fecal CA-derived BA excretion and increased the bile flow, which corroborates previous studies (20, 21). The increased bile flow may result from the osmotic effect of the non-significantly increased BA production rate, which is probably to compensate for the increased fecal BA excretion. In addition, colesevelam largely decreased levels of both hepatic and plasma cholesterol, which can be explained by the fact that cholesterol is the main substrate for BA production (8).

Since β3-AR agonism increased plasma levels of BAs, particularly CA-derived BAs, and decreased fecal BA excretion, we reasoned that β3-AR agonism induces reabsorption of BAs from the gut, which could be effectively inhibited by colesevelam. Indeed, colesevelam on top of β3-AR agonism largely increased fecal excretion of BAs, and in particular CA-derived BAs. As a consequence of increased fecal BA excretion, plasma BA levels were normalized upon colesevelam treatment on top of β3-AR agonism, although this did not reach significance, most probably due to lack of power. The fact that colesevelam on top of β3-AR agonism still lowered hepatic cholesterol to similar levels as reached by

colesevelam alone indicates that the effect of colesevelam on hepatic cholesterol levels is stronger than the β3-AR agonism-mediated effects.

Unexpectedly, prolonged β3-AR agonism caused hepatic inflammation as evidenced by an increased number of inflammatory foci and increased mRNA expression of T-cell markers. Theoretically, hepatic inflammation may be related to hepatic cholesterol accumulation, which can cause cellular toxicity and promote a pro-inflammatory response (22). Alternatively, elevated hepatic BA levels can also directly cause hepatic inflammation (23). We observed that colesevelam on top of β3-AR agonism did not reduce hepatic inflammation despite improved hepatic cholesterol levels and plasma BA levels. This suggests that hepatic inflammation is not induced by cholesterol accumulation in the liver as induced by β3-AR agonism, but is rather a direct effect of β3-AR agonism. Indeed, β3-AR agonism increases circulating monocyte counts [*Hoeke et al*., unpublished], and has been described to activate macrophages (24). These direct effects of β3-AR agonism on immune cells may be caused by cross reactivity of CL316,243 with the β2-AR that is expressed on immune cells (25). Alternatively, CL316,243 may activate the β3-AR that is expressed by niche cells in the bone marrow, which are responsible for differentiation of immune cells. Upon activation of the β3-AR, circulating immune cells increase (26) and these cells may also migrate towards the liver, thereby inducing hepatic inflammation.

The combination of colesevelam and β3-AR agonism not only improved BA metabolism and hepatic cholesterol levels, but also improved TG metabolism. This combination seemed to even further reduce plasma TG levels and gWAT weight as compared to β3-AR agonism alone. Reduced plasma TG levels may be due to both increased TG-derived FA combustion upon β3-AR agonism and reduced dietary FA uptake by the intestines upon colesevelam treatment, as this has been described before for the BA sequestrant colestilan (27). As a consequence of reduced FA availability, FA are released from WAT in order to provide enough substrate for activated BAT, which is completely in line with the additive effect of colesevelam to β3-AR agonism in the lowering of gWAT weight.

Preclinical studies showed that both β3-AR agonism alone and colesevelam alone not only reduce plasma cholesterol levels, but also atherosclerosis development (4, 21). Importantly, we now showed that colesevelam on top of β3-AR agonism further reduces plasma non-HDL-C levels as compared to β3-AR agonism alone. Although we did not assess atherosclerosis development, these data strongly suggest that the combination

of colesevelam and β3-AR agonism may further reduce atherosclerosis development as compared to β3-AR agonism alone. This would be of high relevance from a clinical perspective. The β3-AR is present in humans, its activation increases BAT activity and resting energy expenditure (28), and high BAT activity is associated with a reduced risk of CVD events (29). In addition, BA sequestrants reduce coronary heart disease (30) and coronary artery lesions in humans (31). Altogether, BAT activation combined with colesevelam treatment could be a valuable strategy for the treatment of CVD in humans and future studies should therefore focus on the potential anti-atherogenic effects of this combination therapy.

In conclusion, we showed that prolonged β3-AR agonism increases both biliary BA secretion as well as BA reabsorption from the gut, resulting in decreased fecal BA excretion. This sequence of events causes downregulation of hepatic BA synthesis and accumulation of hepatic cholesterol. Concomitant treatment with colesevelam increases fecal BA excretion, reverses the β3-AR agonism-mediated hepatic cholesterol accumulation, and further lowers plasma non-HDL-C levels as compared to β3-AR agonism alone. Based on these data, we anticipate that colesevelam may enhance the beneficial effects of β3-AR agonism on atherosclerosis development, which should be shown in future studies.

DISCLOSURES

The authors have nothing to disclose.

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REFERENCES

1. Labbe, S. M., A. Caron, I. Bakan, M. Laplante, A. C. Carpentier, R. Lecomte, and D. Richard, In vivo measurement of energy substrate contribution to cold-induced brown adipose tissue thermogenesis. *FASEB J.* 2015. **29:** 2046-2058.
2. Ouellet, V., S. M. Labbe, D. P. Blondin, S. Phoenix, B. Guerin, F. Haman, E. E. Turcotte, D. Richard, and A. C. Carpentier, Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. *J. Clin. Invest.* 2012. **122:** 545-552.
3. Hoeke, G., S. Kooijman, M. R. Boon, P. C. Rensen, and J. F. Berbee, Role of Brown Fat in Lipoprotein Metabolism and Atherosclerosis. *Circ. Res.* 2016. **118:** 173-182.
4. Berbee, J. F., M. R. Boon, P. P. Khedoe, A. Bartelt, C. Schlein, A. Worthmann, S. Kooijman, G. Hoeke, I. M. Mol, C. John, C. Jung, N. Vazirpanah, L. P. Brouwers, P. L. Gordts, J. D. Esko, P. S. Hiemstra, L. M. Havekes, L. Scheja, J. Heeren, and P. C. Rensen, Brown fat activation reduces hypercholesterolaemia and protects from atherosclerosis development. *Nat. Commun.* 2015. **6:** 6356.
5. Khedoe, P. P., G. Hoeke, S. Kooijman, W. Dijk, J. T. Buijs, S. Kersten, L. M. Havekes, P. S. Hiemstra, J. F. Berbee, M. R. Boon, and P. C. Rensen, Brown adipose tissue takes up plasma triglycerides mostly after lipolysis. *J. Lipid Res.* 2015. **56:** 51-59.
6. Hoeke, G., Y. Wang, A. D. Van Dam, I. M. Mol, E. Gart, H. G. Klop, S. M. Van Den Berg, E. H. Pieterman, H. M. G. Princen, A. K. Groen, P. C. N. Rensen, J. F. P. Berbee, and M. R. Boon, Atorvastatin accelerates clearance of lipoprotein remnants generated by activated brown fat to further reduce hypercholesterolemia and atherosclerosis. *Atherosclerosis* 2017. **267:** 116-126.
7. Bartelt, A., C. John, N. Schaltenberg, J. F. P. Berbee, A. Worthmann, M. L. Cherradi, C. Schlein, J. Piepenburg, M. R. Boon, F. Rinninger, M. Heine, K. Toedter, A. Niemeier, S. K. Nilsson, M. Fischer, S. L. Wijers, W. Van Marken Lichtenbelt, L. Scheja, P. C. N. Rensen, and J. Heeren, Thermogenic adipocytes promote HDL turnover and reverse cholesterol transport. *Nat. Commun.* 2017. **8:** 15010.
8. Kuipers, F., V. W. Bloks, and A. K. Groen, Beyond intestinal soap--bile acids in metabolic control. *Nat. Rev. Endocrinol.* 2014. **10:** 488-498.
9. Thomas, C., R. Pellicciari, M. Pruzanski, J. Auwerx, and K. Schoonjans, Targeting bile-acid signalling for metabolic diseases. *Nat. Rev. Drug Discov.* 2008. **7:** 678-693.
10. Dawson, P. A. and S. J. Karpen, Intestinal transport and metabolism of bile acids. *J. Lipid Res.* 2015. **56:** 1085-1099.
11. Bijland, S., E. J. Pieterman, A. C. Maas, J. W. Van Der Hoorn, M. J. Van Erk, J. B. Van Klinken, L. M. Havekes, K. W. Van Dijk, H. M. Princen, and P. C. Rensen, Fenofibrate increases very low density lipoprotein triglyceride production despite reducing plasma triglyceride levels in APOE*3-Leiden. CETP mice. *J. Biol. Chem.* 2010. **285:** 25168-25175.
12. De Haan, W., J. De Vries-Van Der Weij, J. W. Van Der Hoorn, T. Gautier, C. C. Van Der Hoogt, M. Westerterp, J. A. Romijn, J. W. Jukema, L. M. Havekes, H. M. Princen, and P. C. Rensen, Torcetrapib does not reduce atherosclerosis beyond atorvastatin and induces more proinflammatory lesions than atorvastatin. *Circulation* 2008. **117:** 2515-2522.
13. Van Vlijmen, B. J., A. M. Van Den Maagdenberg, M. J. Gijbels, H. Van Der Boom, H. Hogenesch, R. R. Frants, M. H. Hofker, and L. M. Havekes, Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J. Clin. Invest.* 1994. **93:** 1403-1410.
14. Westerterp, M., C. C. Van Der Hoogt, W. De Haan, E. H. Offerman, G. M. Dallinga-Thie, J. W. Jukema, L. M. Havekes, and P. C. Rensen, Cholesteryl ester transfer protein decreases high-density lipoprotein and severely aggravates atherosclerosis in APOE*3-Leiden mice. *Arterioscler. Thromb. Vasc. Biol.* 2006. **26:** 2552-2559.
15. Hulzebos, C. V., L. Renfurm, R. H. Bandsma, H. J. Verkade, T. Boer, R. Boverhof, H. Tanaka, I. Mierau, P. J. Sauer, F. Kuipers, and F. Stellaard, Measurement of parameters of cholic acid kinetics in plasma using a microscale stable isotope dilution technique: application to rodents and humans. *J. Lipid Res.* 2001. **42:** 1923-1929.
16. Hulzebos, C. V., H. Wolters, T. Plosch, W. Kramer, S. Stengelin, F. Stellaard, P. J. Sauer, H. J. Verkade, and F. Kuipers, Cyclosporin a and enterohepatic circulation of bile salts in rats: decreased cholate

synthesis but increased intestinal reabsorption. *J. Pharmacol. Exp. Ther.* 2003. **304:** 356-363.
17. Van Der Veen, J. N., T. H. Van Dijk, C. L. Vrins, H. Van Meer, R. Havinga, K. Bijsterveld, U. J. Tietge, A. K. Groen, and F. Kuipers, Activation of the liver X receptor stimulates trans-intestinal excretion of plasma cholesterol. *J. Biol. Chem.* 2009. **284:** 19211-19219.
18. Alnouti, Y., I. L. Csanaky, and C. D. Klaassen, Quantitative-profiling of bile acids and their conjugates in mouse liver, bile, plasma, and urine using LC-MS/MS. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2008. **873:** 209-217.
19. Worthmann, A., C. John, M. C. Ruhlemann, M. Baguhl, F. A. Heinsen, N. Schaltenberg, M. Heine, C. Schlein, I. Evangelakos, C. Mineo, M. Fischer, M. Dandri, C. Kremoser, L. Scheja, A. Franke, P. W. Shaul, and J. Heeren, Cold-induced conversion of cholesterol to bile acids in mice shapes the gut microbiome and promotes adaptive thermogenesis. *Nat. Med.* 2017. **23:** 839-849.
20. Herrema, H., M. Meissner, T. H. Van Dijk, G. Brufau, R. Boverhof, M. H. Oosterveer, D. J. Reijngoud, M. Muller, F. Stellaard, A. K. Groen, and F. Kuipers, Bile salt sequestration induces hepatic de novo lipogenesis through farnesoid X receptor- and liver X receptor alpha-controlled metabolic pathways in mice. *Hepatology* 2010. **51:** 806-816.
21. Meissner, M., H. Wolters, R. A. De Boer, R. Havinga, R. Boverhof, V. W. Bloks, F. Kuipers, and A. K. Groen, Bile acid sequestration normalizes plasma cholesterol and reduces atherosclerosis in hypercholesterolemic mice. No additional effect of physical activity. *Atherosclerosis* 2013. **228:** 117-123.
22. Ioannou, G. N., The Role of Cholesterol in the Pathogenesis of NASH. *Trends Endocrinol. Metab.* 2016. **27:** 84-95.
23. Allen, K., H. Jaeschke, and B. L. Copple, Bile acids induce inflammatory genes in hepatocytes: a novel mechanism of inflammation during obstructive cholestasis. *Am. J. Pathol.* 2011. **178:** 175-186.
24. Hadi, T., R. Douhard, A. M. M. Dias, M. Wendremaire, M. Pezze, M. Bardou, P. Sagot, C. Garrido, and F. Lirussi, Beta3 adrenergic receptor stimulation in human macrophages inhibits NADPHoxidase activity and induces catalase expression via PPARgamma activation. *Biochim. Biophys. Acta* 2017. **1864:** 1769-1784.
25. Bellinger, D. L. and D. Lorton, Autonomic regulation of cellular immune function. *Auton. Neurosci.* 2014. **182:** 15-41.
26. Heidt, T., H. B. Sager, G. Courties, P. Dutta, Y. Iwamoto, A. Zaltsman, M. C. Von Zur, C. Bode, G. L. Fricchione, J. Denninger, C. P. Lin, C. Vinegoni, P. Libby, F. K. Swirski, R. Weissleder, and M. Nahrendorf, Chronic variable stress activates hematopoietic stem cells. *Nat. Med.* 2014. **20:** 754-758.
27. Sugimoto-Kawabata, K., H. Shimada, K. Sakai, K. Suzuki, T. Kelder, E. J. Pieterman, L. H. Cohen, L. M. Havekes, H. M. Princen, and A. M. Van Den Hoek, Colestilan decreases weight gain by enhanced NEFA incorporation in biliary lipids and fecal lipid excretion. *J. Lipid Res.* 2013. **54:** 1255-1264.
28. Cypess, A. M., L. S. Weiner, C. Roberts-Toler, E. F. Elia, S. H. Kessler, P. A. Kahn, J. English, K. Chatman, S. A. Trauger, A. Doria, and G. M. Kolodny, Activation of Human Brown Adipose Tissue by a beta3-Adrenergic Receptor Agonist. *Cell Metab.* 2015. **21:** 33-38.
29. Takx, R., A. Ishai, Q. A. Truong, M. H. Macnabb, M. Scherrer-Crosbie, and A. Tawakol, Supraclavicular Brown adipose tissue FDG uptake and cardiovascular disease. *J. Nucl. Med.* 2016. **57:** 1221-1225.
30. The Lipid Research Clinics Coronary Primary Prevention Trial results. II. The relationship of reduction in incidence of coronary heart disease to cholesterol lowering. *JAMA* 1984. **251:** 365-374.
31. Brensike, J. F., R. I. Levy, S. F. Kelsey, E. R. Passamani, J. M. Richardson, I. K. Loh, N. J. Stone, R. F. Aldrich, J. W. Battaglini, D. J. Moriarty, and . Effects of therapy with cholestyramine on progression of coronary arteriosclerosis: results of the NHLBI Type II Coronary Intervention Study. *Circulation* 1984. **69:** 313-324.

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