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Intestinal bile acid reabsorption in health and disease

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CHAPTER

6

Efficient reabsorption of transintestinally excreted cholesterol is a strong determinant for cholesterol disposal in mice

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Abstract

Transintestinal cholesterol excretion (TICE) is a major route for cholesterol elimination from the body and a potential therapeutic target for hypercholesterolemia. The underlying mechanism, however, is largely unclear and its contribution to cholesterol disposal from the body is obscured by the counteracting process of intestinal cholesterol reabsorption. To determine the quantity of TICE independent from its reabsorption, we studied two models of decreased intestinal cholesterol absorption. Cholesterol absorption was inhibited either by ezetimibe or, indirectly, by genetic inactivation of the intestinal apical sodium-dependent bile acid transporter (ASBT, *SLC10A2*). Both ezetimibe treatment and *Asbt* inactivation virtually abrogated fractional cholesterol absorption (from 46% to 4% and 6%, respectively). In either model, fecal neutral sterol excretion and net intestinal cholesterol balance were considerably higher than in control mice (5- and 7-fold, respectively), suggesting that, under physiological conditions, TICE is largely reabsorbed. In both models, the net intestinal cholesterol balance was increased to a similar extent, but was not further increased when the models were combined, suggesting that the effect on cholesterol reabsorption was already maximal under either condition alone. Based on these findings, we hypothesize that inhibition of cholesterol (re)absorption combined with stimulation of TICE will be most effective in increasing cholesterol disposal.

Introduction

Atherosclerosis, leading to coronary artery disease and cerebrovascular accidents, accounts for around 50% of deaths in Westernized countries and its prevalence is increasing in low- and middle-income countries (1,2). The aetiology of atherosclerosis is complex and risk factors are both genetic and environmental. Hypercholesterolemia can contribute to the development of atherosclerosis via accumulation of cholesterol from low-density lipoproteins (LDL-c) in the arterial vessel wall (2,3). Treatment options for hypercholesterolemia and atherosclerosis include lifestyle modifications (i.e. smoking cessation, increasing physical activity and improving diet quality (4,5)), as well as drugs targeted at cholesterol metabolism. The most widely prescribed class of drugs are statins that inhibit hepatic cholesterol synthesis. Unfortunately, statins only reduce the cardiovascular disease risk by 15-37% (6). Novel proprotein convertase subtilsinkexin type 9 (PCSK9) inhibitors show promising results, but also limitations (7). Therefore, more effective or adjunct treatments are needed for the prevention and treatment of atherosclerosis.

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Cholesterol homeostasis in the body encompasses a highly regulated balance between cholesterol intake, *de novo* synthesis, and disposal, mainly via the feces as neutral sterols (NS; cholesterol and its metabolites produced by intestinal microbiota) or as bile acids (BAs). BAs are synthetized from cholesterol in the liver, secreted into the bile and predominantly stored in the gallbladder. Following a meal and gallbladder emptying, BAs are secreted via the bile into the duodenum, where they aid in the absorption of fat, cholesterol and fat-soluble vitamins. Under physiological conditions, about 95% of intestinal BAs are reabsorbed each cycle, mainly by ileal enterocytes via the apical sodium-dependent bile acid transporter (ASBT, *SLC10A2*), and transported back to the liver. This mechanism of enterohepatic circulation is tightly regulated by the BA-activated nuclear farnesoid X receptor (FXR) in both the liver and intestine (8).

Inducing fecal excretion of both NS and BAs has been used as a strategy to lower plasma cholesterol levels. Blocking cholesterol absorption by inhibiting the main intestinal cholesterol transporter, the Niemann-Pick C1-Like 1 (NPC1L1), increases fecal cholesterol excretion and hepatic LDL-receptor (LDLR) expression, and is used as adjunct therapy to statins to further reduce plasma LDL-c levels and improve cardiovascular outcomes (9). Increasing cholesterol excretion from the body can also be achieved by targeting BA homeostasis. BA sequestrants bind

BAs inside the intestinal lumen, thereby preventing their reabsorption, ultimately resulting in increased fecal BA excretion and a compensatory increase in BA synthesis from cholesterol (10). BA sequestrants have been shown to effectively lower plasma LDL-c levels in animal models as well as humans (11). ASBT inhibition works via a similar mechanism and has also been shown to reduce hypercholesterolemia and atherosclerosis in several animal models (12–15). Inhibition of ASBT reduces reabsorption of BAs and, similar to BA sequestrants, this increases their fecal excretion which is compensated by increased hepatic synthesis from cholesterol. Interrupting the enterohepatic circulation by inhibiting ASBT decreases the BA pool (i.e. the total amount of BAs present in the enterohepatic circulation), because the induction of synthesis cannot completely compensate for the increased fecal loss (16). This results in a decreased availability of BAs in the intestinal lumen for the solubilisation of cholesterol, thereby lowering intestinal cholesterol absorption (17).

Recently, a major non-biliary pathway that contributes to the fecal excretion of cholesterol has been identified. This pathway, known as transintestinal cholesterol excretion (TICE), is present both in mice and humans (18,19). The molecular mechanism underlying TICE has not been fully elucidated. However, TICE is at least partly dependent on cholesterol transport by the ATP-binding cassette sub-family G members 5 and 8 (ABCG5/8) (20–22). Originally, Van der Velde *et al.* quantified TICE directly in intestinal perfusion studies (23). In subsequent, more physiological studies, TICE was measured indirectly by subtracting dietary input and biliary cholesterol output from fecal neutral sterol (NS) excretion (reviewed in (24,25)). This yields a minimum estimation of TICE, since it is not clear to what degree reabsorption of transintestinally-excreted cholesterol influenced the contribution to fecal cholesterol excretion. The notion that reabsorption had a potential influence could be concluded from experiments with ezetimibe, an NPC1L1 inhibitor, which further increased fecal NS excretion beyond biliary and dietary input (19,26,27). Various other conditions, including high-fat diet feeding, liver X receptor (LXR) activation and intestinal FXR activation have been implied to affect TICE, but so far the possible role of reabsorption of cholesterol originating from TICE has not been addressed rigorously (21–23).

In the current study, we investigated the contribution of cholesterol (re)absorption of intestinally excreted cholesterol by using two models of impaired cholesterol (re)absorption. Firstly, we inhibited intestinal cholesterol (re)absorption by using ezetimibe, which inhibits NPC1L1. Secondly, we used *Asbt*^{-/-} mice which

display a partial impairment in cholesterol (re)absorption through reduction of the BA pool (17). In both models, we quantitated cholesterol fluxes and measured fractional cholesterol absorption. Lastly, we combined both models to determine the combined effect of ezetimibe and ASBT inhibition on cholesterol disposal from the body.

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Methods

Animals

Asbt^{-/-} mice and *wildtype* (WT) littermates on a C57BL/6 were originally generated by P.A. Dawson (Emory University, Atlanta, GA) and bred at the UMCG animal facility. Male mice were used at ages between 10-18 weeks. Mice were conventionally housed in individual cages in a temperature- and light-controlled facility with a 12-hour light-dark cycle. The mice had *ad libitum* access to water and maintenance laboratory chow (macronutrient ratio as % of total calories: fat: 7.5%, proteins: 17.5%, carbohydrates: 75%) containing 0.008% cholesterol (RM1 FG, Special Diet Services, Witham Essex, England), with or without ezetimibe (0.005%, 50mg/kg chow) (Ezetrol, Pharmacy UMCG, Groningen, The Netherlands).

Animal experiments were approved by the Ethics Committee for Animal Experiments of the University of Groningen. All experiments were performed in accordance with relevant guidelines and regulations (including laboratory and biosafety regulations).

Cholesterol flux measurements

Mice received the ezetimibe-enriched diet for 3 weeks. Cholesterol fluxes were measured in the last 10 days by using a dual stable isotope tracer method as described (28). The experimental setup is shown in **Figure 1**. Three days prior to the start of the experiment, bloodspots and 24-hour feces were collected for baseline measurements and food intake and body weight were measured. On day 0, the mice were anaesthetized with isoflurane and given a retro-orbital injection of 0.3mg D_5-cholesterol (Medical Isotopes Inc., Pelham, NH, USA) dissolved in 150µl Intralipid 20% (Fresenius Kabi, Den Bosch, The Netherlands) and an oral gavage of 0.6mg D_7-cholesterol (Cambridge Isotope Laboratories, Inc, Andover, MA, USA) dissolved in 200µl medium chain triglyceride oil. Bloodspots were collected at the time points 3, 6, 12, 24, 48, 72, 96, 120, 144 and 168 hours after labelled cholesterol administration. At the time point 168h (day 8), mice received water containing 2% of 1-[13C]acetate until termination, and bloodspots were collected 24, 32, 48 and 72 hours after starting the 1-[13C]acetate. Body weight and food intake were determined and feces were collected daily from day 0 to day 10 (figure 1).

On day 10, the mice were anesthetized by intraperitoneal injection of a mixture of Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg). The gallbladder was cannulated as previously described (29). Bile collected in the first 5 minutes was discarded to avoid collection of concentrated bile. After this first 5 min, bile was collected for 20 minutes in pre-weighed tubes with the mice placed in a

humidified incubator (37ºC) to maintain body temperature. Blood was obtained via cardiac puncture. The small intestines were flushed with ice cold phosphate buffered saline (PBS) containing a protease inhibitor (cOmplete, Roche, Mannheim, Germany) and cut in three segments of equal length, the middle-piece from each segment was excised for gene analysis. All intestinal segments were immediately snap-frozen in liquid nitrogen.

Figure 1. **Experimental schedule, adapted from** (21).

Bile acid and neutral sterol measurements

Neutral sterols (cholesterol and its bacterial metabolites in fecal samples) were extracted from 50mg of air-dried, ground fecal samples as described by Ronda *et al.* (28). Briefly, feces was heated for two hours at 80ºC with a mixture of 1M sodium hydroxide and methanol (1:3). Neutral sterols were then extracted 2 times with 2ml petroleum ether and derivatised with BSTFA-pyridine-TMCS (5:5:0.1). Bile acids were extracted from feces with Sep-Pak C-18 columns, methylated with methanol/acetyl chloride (20:1) and derivatised with BSTFA-pyridine-TMCS (5:5:0.1). Both neutral sterols and bile acids were measured by gas chromatography (GC) as previously described (30). The total amount of bile acids or neutral sterols was calculated as the sum of the individual species.

For biliary bile acid measurements, bile samples were diluted 1000-fold with Milli-Q water. Samples were centrifuged at 15800 x g and the supernatant poured into a clean glass tube. The fluid was evaporated under nitrogen at 40°C. Before measuring, samples were reconstituted in 200 µL 50% methanol in water, vortexed for 60 s and centrifuged for 3 min at 1800 x g. The supernatant was transferred into a 0,2 µm spin-filter and centrifuged at 2000 x g for 10 min. After filtering, the samples were transferred into vials and analyzed (10 µL injection volume). For the quantitative determination of bile acids we used a Nexera X2 Ultra High Performance Liquid Chromatography system (SHIMADZU, Kyoto, Japan), coupled to a SCIEX QTRAP 4500 MD triple quadrupole mass spectrometer (SCIEX, Framingham, MA, USA) (UHPLC-MS/MS). The LC-MS/MS system is controlled by Analyst MD 1.6.2 software.

Biliary lipids were extracted from 15µl of bile according to Bligh and Dyer (31) and, subsequently, biliary cholesterol was derivatised with BSTFA-pyridine-TMCS (5:5:0.1) for GC measurement (28).

Net non-hepatobiliary cholesterol excretion was calculated as [fecal neutral sterol output - (dietary cholesterol intake + hepatobiliary secretion)]. Cholesterol synthesis and pool size were calculated as described in (28).

Hepatic and plasma lipids

Livers were mechanically ground in liquid nitrogen. Liver lipids were extracted from 15% homogenates in PBS according to Bligh and Dyer (31). Subsequently, liver total and free cholesterol and triglyceride levels were determined using commercially available reagents (DiaSys Diagnostic Systems, Holzheim, Germany and Roche Diagnostics, Mannheim, Germany). Plasma triglycerides, total cholesterol and free cholesterol were determined spectrophotometrically using the same kits. For plasma lipoprotein measurements, blood from individual mice was pooled for each experimental group. Plasma lipoproteins were fractionated using fast protein liquid chromatography (FPLC) on a Superose - 6 10/300 GL column (GE Healthcare, Uppsala, Sweden). Cholesterol and triglyceride concentrations of fractions were determined using commercially available reagents (DiaSys Diagnostic Systems and Roche Diagnostics).

Gene expression analysis

Gene expression analysis was performed in liver and duodenum. Total RNA was isolated with TRI-Reagent (Sigma, St. Louis, MO, USA) and quantified by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). cDNA synthesis was

performed from 1μg of total RNA. Primers were designed with Primer-BLAST and optimized for use with SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany) (maximum product size 150 nucleotides). Real-time qPCR analysis was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher, Darmstadt, Germany). Gene expression levels were normalized to *36b4*. Results were quantified using the comparative Ct method.

Statistical analyses

Unless otherwise stated, data are presented as Tukey plots where boxes represent the median with interquartile range (IQR) and whiskers extend to the largest value or 1.5 times the IQR if the largest value extends that. Statistical analyses were performed and graphs were created using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Differences between groups were assessed by 2-way ANOVA using Tukey's post-hoc test. Significance is indicated as $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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Results

Assessment of cholesterol (re)absorption in ezetimibe-treated mice

We aimed to estimate the amount of cholesterol entering the intestine via TICE. To avoid interference of potential intestinal reabsorption, we first applied ezetimibe treatment and determined fractional cholesterol absorption using a dual stable isotope labelling approach (**Fig. 2A**). Ezetimibe prevents intestinal cholesterol absorption through inhibition of the internalization of the cholesterol transporter NPC1L1, which is required for intestinal cholesterol absorption in the small intestinal epithelium (32). Ezetimibe treatment virtually abrogated fractional cholesterol absorption (from 46% in untreated controls to 4% upon ezetimibe treatment, *P* < 0.001, **Fig. 2A**). In line with earlier studies (22), ezetimibe treatment increased fecal neutral sterol (NS) excretion 4-fold in WT mice (**Fig.2B**). Theoretically, there are three mechanisms possible for an increased fecal NS: 1) increased influx of cholesterol into the intestine (either via the bile, the diet or TICE), 2) decreased intestinal (re)absorption, or 3) a combination of these. Since cholesterol absorption is virtually abrogated by ezetimibe treatment, cholesterol (re)absorption can be considered minimal, leaving the three remaining fluxes as possibly causing the increased fecal NS: biliary secretion, dietary cholesterol intake and/or TICE (**Fig. 3A**). The resultant flux of excretion (i.e. TICE) minus (re)absorption was defined as net intestinal (cholesterol) balance. (**Fig. 3A**). Based on the difference between fecal NS excretion and the dietary and biliary cholesterol influx into the intestine, the net intestinal balance in ezetimibe-treated mice was estimated at 39 µmol/24h/100gBW (**Fig. 3B-C**). The estimated net intestinal cholesterol balance thereby largely exceeds the biliary and the dietary influx of cholesterol into the intestine. These data indicate that the profound increase in fecal NS is apparently for the largest part due to TICE, that is not reabsorbed upon ezetimibe treatment.
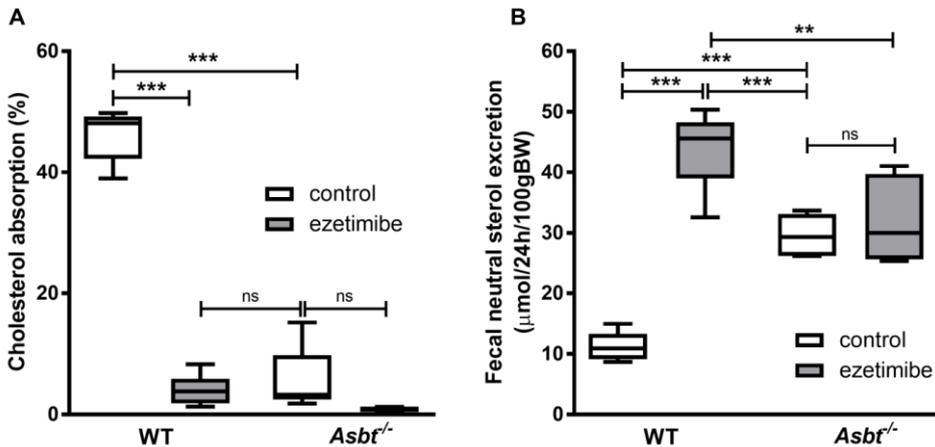
Figure 2. Intestinal cholesterol absorption and fecal neutral sterol excretion in WT and *Asbt*^{-/-} mice with and without ezetimibe treatment. Fractional cholesterol absorption (A) measured by stable dual isotope method. Total neutral sterol excretion in feces (B); n=5-6 per group.

Assessment of cholesterol (re)absorption in Asbt^{-/-} *mice*

Theoretically, the results obtained in ezetimibe-treated mice could be specific for this mechanism of inhibition of cholesterol absorption and thereby not generalizable to other conditions of decreased cholesterol (re)absorption. We therefore performed similar experiments in another mouse model of decreased cholesterol absorption and increased fecal NS excretion, the *Asbt*^{-/-} mice. The fractional cholesterol absorption in *Asbt*^{-/-} mice was strongly decreased, to a similar level of that of ezetimibe-treated WT mice (6% vs 4%, *P* = ns, **Fig. 2A**). In agreement with previous reports, *Asbt*^{-/-} mice had a 3-fold increased fecal NS excretion as compared to WT controls (*P* < 0.001, **Fig. 2B**) (17). Under these conditions of virtually no cholesterol (re)absorption, the net intestinal cholesterol balance was 26 μmol/24h/100gBW, slightly lower but in the same range as intestinal cholesterol balance in ezetimibe-treated WT mice (*P* < 0.01, **Fig. 3B-C**). TICE was several fold larger than the biliary and dietary cholesterol influx into the intestine and, again, the increased fecal NS fraction could largely be attributed to non-reabsorbed cholesterol originating from TICE.

We then investigated whether combining the two mechanisms of inhibiting (re)absorption would further affect these cholesterol fluxes across the intestine. Treatment of *Asbt*^{-/-} mice with ezetimibe, however, did not result in a significant (further) reduction of cholesterol absorption compared to untreated *Asbt*^{-/-} mice (1% vs 6%, *P* = ns, **Fig. 2A**), nor did it affect either the fecal NS excretion in *Asbt*^{-/-} mice (**Fig. 2B**), the biliary or dietary cholesterol influx or the calculated net

intestinal cholesterol balance (**Fig. 3B-C**). This observation establishes impaired cholesterol (re)absorption as the mechanism underlying the increased fecal NS excretion in (untreated) *Asbt*^{-/-} mice.
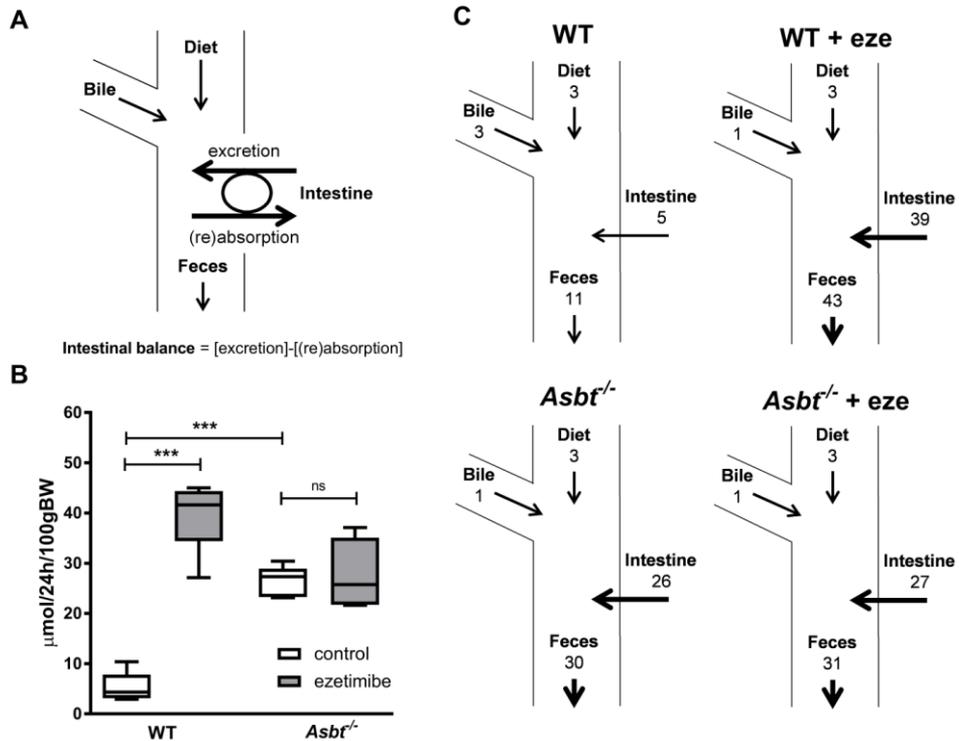

Figure 3. Proposed model for intestinal cholesterol fluxes and calculated net intestinal cholesterol balance for WT and *Asbt*^{-/-} mice with and without ezetimibe treatment. Net intestinal (cholesterol) balance represents [intestinal excretion (i.e. TICE)]-[intestinal (re)absorption] (A), calculated net intestinal cholesterol balance (B) and resultant cholesterol fluxes according to the proposed model (C). Values in µmol/24h/100gBW; n=5-6 per group.

The mechanism of decreased cholesterol (re)absorption in Asbt$^{-/-}$ mice

For ezetimibe, the mechanism of inhibition of cholesterol (re)absorption has been directly related to inhibition of NPC1L1, the main protein responsible for cholesterol absorption (33). For *Asbt*$^{-/-}$ inactivation, however, the mechanism of decreased cholesterol (re)absorption has been less clear (34). Intestinal cholesterol absorption is strongly dependent on the intestinal availability of (hydrophobic) BAs (35). Decreased biliary BA secretion, due to increased intestinal BA loss and contraction of the BA pool, could underlie the decreased cholesterol (re)absorption. We therefore determined whether the interrupted enterohepatic circulation in *Asbt*$^{-/-}$ mice quantitatively and/or qualitatively affected biliary BA secretion and fecal BA excretion. Fecal BA excretion was about 3-fold higher in *Asbt*$^{-/-}$ mice compared to WT mice (25 vs. 8 µmol/24h/100gBW, $P < 0.001$, **Fig. 4A**). At the same time, mRNA level of cholesterol 7 alpha-hydroxylase (*Cyp7a1*), the rate limiting enzyme in the conversion of cholesterol to BAs, was increased 7-fold in *Asbt*$^{-/-}$ mice as compared to WT mice (**Fig. S1**). Ezetimibe treatment did not change fecal BA excretion in WT mice, but slightly increased BA excretion in *Asbt*$^{-/-}$ mice (33 vs 25 µmol/24h/100gBW, $P < 0.05$, **Fig. 4A**). In line with interruption of the enterohepatic circulation of BAs and the subsequent contraction of the BA pool, the biliary secretion of BAs was strongly decreased in *Asbt*$^{-/-}$ mice as compared to WT controls (-86%, $P < 0.001$, **Fig. 4B**). Ezetimibe did not significantly affect total biliary BA secretion in WT mice or *Asbt*$^{-/-}$ mice (**Fig. 4B**). The composition of biliary BAs was more hydrophobic in *Asbt*$^{-/-}$ mice compared to controls, as quantified by an increased Heuman hydrophobicity index (+0.2 vs -0.2, $P < 0.001$, **Fig. 4C**) (36). The increase in hydrophobicity could be attributed to a fractional increase of taurodeoxycholic acid (TDCA) (47% vs 4%, $P < 0.001$, **Fig. 4D**) and subsequent decrease in taurocholic acid (TCA) (35 vs 67%, $P < 0.001$, **Fig. 4D**) and tauro-β-muricholic acid (TβMCA) (2% vs 21%, $P < 0.001$, **Fig 4D**) in *Asbt*$^{-/-}$ mice compared to WT mice. Ezetimibe did not affect fecal BA composition (data not shown), biliary hydrophobicity (**Fig 4C**) or biliary BA profile (**Fig 4D**) in either WT or *Asbt*$^{-/-}$ mice .

Not only the absorption of cholesterol, but also that of dietary fatty acids was decreased in *Asbt*$^{-/-}$ mice and this was slightly ameliorated by ezetimibe (**Fig. 4E**). Together, these findings indicate that the abrogated intestinal cholesterol absorption in *Asbt*$^{-/-}$ mice primarily resulted from strongly reduced biliary BA secretion. Apparently, the decreased biliary BA secretion could not be compensated for by a more hydrophobic BA composition, despite the notion that hydrophobic BAs are more effective in aiding the micellar solubilization and subsequent absorption of cholesterol (35,37).

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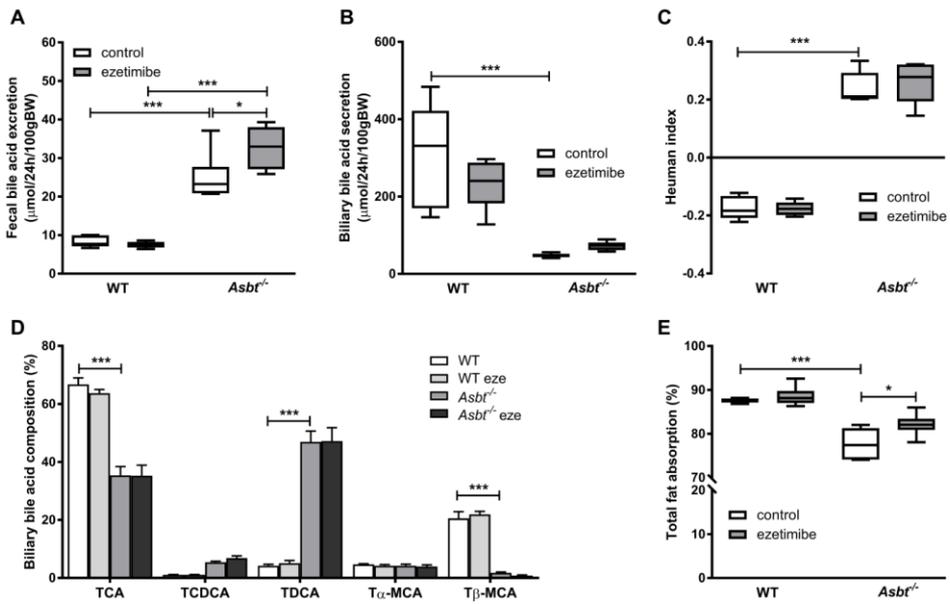
Figure 4. Changes in fecal and biliary bile acids and intestinal fat absorption upon ezetimibe treatment and *Asbt* deletion in mice. Total fecal bile acid excretion (A), total biliary bile acids secretion (B), biliary hydrophobicity index based on Heuman values (C), biliary bile acid composition (D) and intestinal fatty acid absorption (E); n=5-6 per group.

Intestinal and hepatic mRNA expression of genes involved in cholesterol homeostasis

Theoretically, the decreased cholesterol absorption in *Asbt*^{-/-} mice could be due to downregulation of *Npc1l1* expression in the duodenum, but the unaffected steady state mRNA levels did not support this possibility (**Fig. 5A**). A reduction in cholesterol absorption can affect intracellular cholesterol concentrations, which is sensed by the liver X receptor (LXR). Therefore, we measured the expression of LXR target genes, the ATP-binding cassette (ABC) subfamily A member 1 (*Abca1*) and ABC subfamily G members 5 and 8 (*Abcg5/8*). Abcg5/8 promote cholesterol efflux from the cell and are known to be crucial for TICE (24,38). *Abca1* was decreased in WT ezetimibe-treated mice compared to WT controls and in *Asbt*^{-/-} mice compared to WT controls (**Fig. 5A**). *Abcg5/8* showed a similar trend to *Abca1,* but the difference did not reach statistical significance (**Fig. 5A**).

To assess the consequences of decreased intestinal cholesterol (re)absorption and increased BA synthesis on hepatic cholesterol homeostasis, we also measured LXR target genes in the liver (**Fig. 5B**). Levels of mRNA of the low density lipoprotein receptor 1 (*Ldlr1*), of *Abca1* and of the sterol regulatory element-binding protein-1c (*Srebp1c*) were not statistically different.

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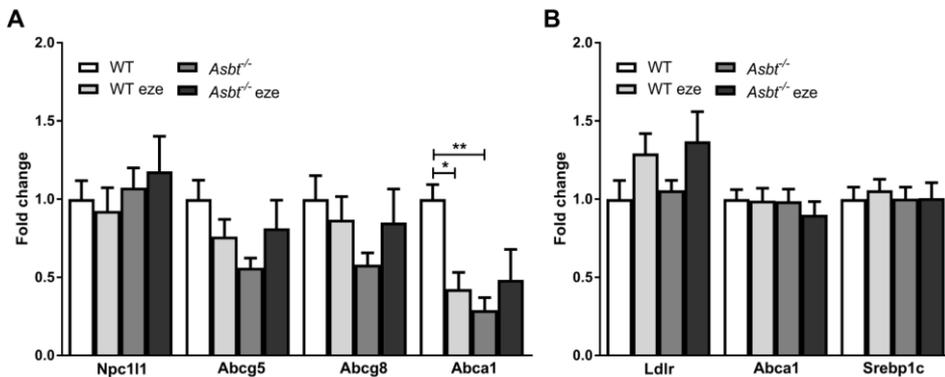
Figure 5. Expression of genes related to cholesterol homeostasis in WT and *Asbt*^{-/-} mice with and without ezetimibe treatment. Duodenal expression of genes related to cellular cholesterol homeostasis (A) and hepatic expression of LXR target genes (B), mean ± SEM; n=5-6 per group.

Effects of ezetimibe treatment and Asbt inactivation on total sterol excretion and cholesterol synthesis

Disposal of cholesterol from the body is achieved via excretion, either as NS or, after conversion, as BAs. We calculated the total sterol balance from total fecal sterol output and dietary sterol input without correction for *de novo* synthesis, which will be assessed later (schematically represented in **Fig. 6**). As this model includes all cholesterol fluxes into the intestinal lumen, it represents a total sterol input-output balance over the intestine. Dietary sterol input (composed of dietary cholesterol ingestion) was similar across the groups (**Fig. 6A**). Total fecal sterol output, calculated as the sum of NS and BA output, was elevated in *Asbt*$^{-/-}$ control and WT ezetimibe-treated mice to a similar degree upon comparison to WT controls (**Fig. 6B**). In *Asbt*$^{-/-}$ mice, ezetimibe treatment further augmented total sterol excretion in the form of BAs (by ~17%, *P* < 0.01, **Fig. 6B**). All mice displayed a negative intestinal sterol balance, implying that sterol output was greater than input **(Fig. 6C)**. Total intestinal sterol balance in *Asbt*$^{-/-}$ and ezetimibe-treated WT mice was similarly negative (implying more disposal than input), although *Asbt*$^{-/-}$ mice excreted more sterols in the form of BAs, whereas WT ezetimibe-treated mice excreted more in the form of NS. Ezetimibe treatment in *Asbt*$^{-/-}$ mice caused a further decrease in the total sterol balance.

To maintain a steady state in the body, a negative intestinal sterol balance needs to be compensated for by increased cholesterol synthesis. Indeed, enhancing cholesterol disposal via inhibition of reabsorption induces a compensatory increase in *de novo* cholesterol synthesis (39). We determined cholesterol synthesis in the four experimental groups, using ^{13}C-acetate labelled drinking water (28). **Fig. 7A** shows the fractional contribution of newly synthesized cholesterol in plasma. Cholesterol synthesis was increased to a similar extent in *Asbt*$^{-/-}$ and ezetimibe-treated WT mice, compared to untreated WT mice. The changes in cholesterol synthesis rates coincided with a similar trend towards higher gene expression of HmG-CoA reductase (*Hmgcr*) in both *Asbt*$^{-/-}$ groups and WT ezetimibe-treated mice (**Fig. 7B**). Ezetimibe treatment in *Asbt*$^{-/-}$ mice further increased cholesterol synthesis compared to *Asbt*$^{-/-}$ controls (**Fig. 7A**). The changes in cholesterol absorption and synthesis did neither affect plasma cholesterol levels, lipoprotein distribution or hepatic cholesterol levels, nor the calculated total body cholesterol pool size (**Fig. S2-S4**).
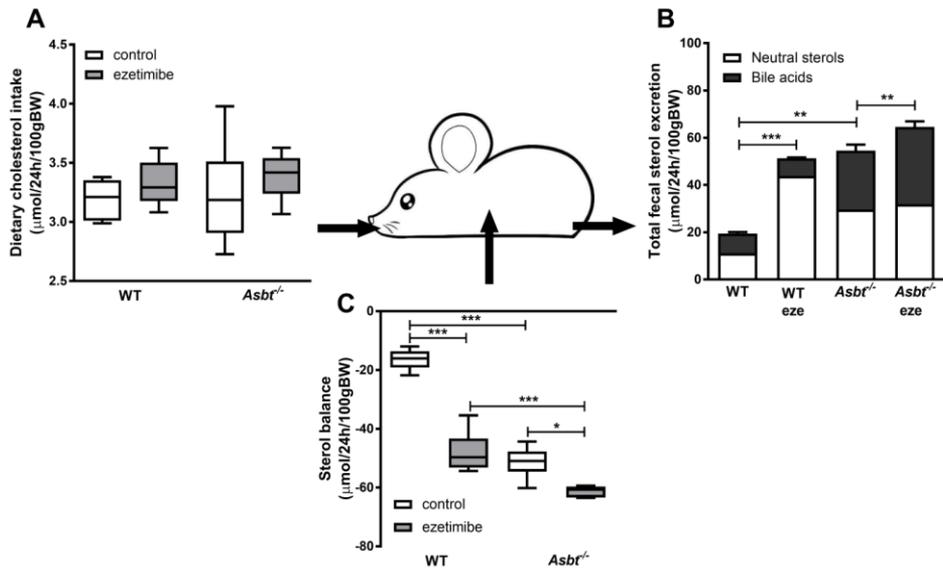
Figure 6. Schematic representation of the total sterol balance over the intestine, calculated via dietary cholesterol intake and total sterol output of WT and *Asbt*^{-/-} mice with and without ezetimibe treatment. Dietary cholesterol intake (A), total fecal sterol excretion (B) and net total sterol balance over the intestine (C); n=5-6 per group.

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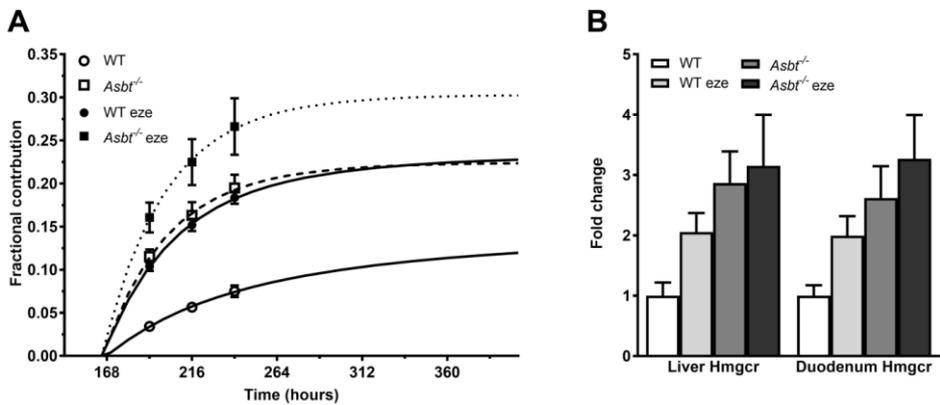
Figure 7. Changes in cholesterol synthesis upon ezetimibe treatment and *Asbt*^{-/-} in mice. Fractional contribution of newly synthesized cholesterol in plasma. Hepatic (A) and duodenal (B) *Hmgcr* gene expression; n=5-6 per group

Discussion

We aimed to estimate to what extent cholesterol entering the intestinal lumen via TICE is reabsorbed, and thus does not contribute to fecal NS excretion. Our data demonstrate that, using two mechanistically different models of impaired cholesterol (re)absorption, the net intestinal cholesterol balance is similarly increased. This indicates that efficient reabsorption of TICE strongly limits the disposal of cholesterol from the body under physiological conditions. Therefore, to enhance cholesterol disposal from the body, strategies to stimulate TICE are expected to be most efficacious when they are combined with simultaneous inhibition of its reabsorption.

Previous studies showed that ezetimibe increases fecal NS excretion beyond what is expected based upon the decrease in absorption of dietary and bile-derived cholesterol (18,19,22). Up to now, it had not been possible to distinguish conclusively whether the increase in fecal NS beyond dietary and biliary input was due to increased TICE or to decreased (re)absorption. In the present study, we used a simplified model based on a net intestinal cholesterol balance, calculated by subtracting dietary and biliary cholesterol input from fecal output (**Fig. 2A**). In this model, the net intestinal cholesterol balance could be induced via either stimulation of TICE, via reduced (re)absorption or via a combination of both. It should be noted that the net intestinal cholesterol balance encompasses the absorption of cholesterol originating from biliary and dietary origin and the loss of cholesterol in the form of shedding of intestinal cells (at least for the part that is not reabsorbed from the intestine). Based on the provided quantitative calculations and on the estimates in the literature (21,23), these individual contributions, however, are much smaller than the excretion and reabsorption of transintestinally excreted cholesterol. Our data show that abrogating cholesterol absorption, either via ezetimibe or *Asbt* inactivation, elevated the net intestinal cholesterol balance to a similar extent. Under the conditions of impaired cholesterol (re)absorption, the net intestinal cholesterol balance is almost completely determined by TICE. Therefore, we argue that blocking intestinal cholesterol absorption results in increased fecal NS excretion primarily via inhibiting the reabsorption of a basal flux of transintestinally excreted cholesterol into the intestine, which, under physiological conditions, would have been mostly reabsorbed. The reabsorption of transintestinally excreted cholesterol seems to be even more efficient than the (re)absorption of cholesterol from dietary or biliary origin. We cannot exclude that the excretion of transintestinally excreted cholesterol at the apical membrane of

intestinal epithelial cells, i.e. close to the site of possible reabsorption, is responsible for this.

The molecular mechanism underlying the TICE is not fully understood. One explanation is based on the notion that there is spontaneous cholesterol transfer from lipoproteins to membranes in various tissues including the intestine (40). High intestinal bile phospholipid concentrations can potentially be involved in TICE due to their cholesterophilic properties (41). Free cholesterol can transfer to phospholipid rich intestinal bile content and subsequently be excreted in the feces. It might be that under physiological conditions there is a basal flux of cholesterol from the blood to the membranes, the intestinal lumen and back. With either ezetimibe or Asbt deficiency, this basal cholesterol flux is interrupted at the level of reabsorption into the enterocyte. Based on this theory, the slight difference in intestinal cholesterol balance between WT mice treated with ezetimibe versus inactivation of *Asbt* (**Fig. 2B**), could be due to the higher biliary phospholipid secretion in the bile of WT ezetimibe treated mice compared to *Asbt*^{-/-} mice (**Fig. S5**).

Another explanation of differences in intestinal cholesterol balance might be found in the different mechanism underlying the reduction of cholesterol absorption in WT ezetimibe treated mice versus *Asbt*^{-/-} mice. It is proposed that cholesterol either from the intestinal lumen or from endogenous sources first has to translocate into the brush border membrane (BBM) before it can be internalized together with NPC1L1 into the enterocyte (27). In *Asbt*^{-/-} mice the absorption defect is likely due to reduced micelle formation: the decreased intestinal availability of micellar BAs prevents luminal cholesterol from (re-)entering the BBM and thereby its internalization by NPC1L1. Blocking NPC1L1 by ezetimibe is not expected to prevent cholesterol from entering the brush border membrane. Thus, there might be more cholesterol in the BBM upon ezetimibe treatment compared to inactivation of *Asbt* resulting in a higher intestinal efflux, partly mediated via Abcg5/8 (19,27), of cholesterol from the BBM to the lumen from both exo- and endogenous sources. This hypothesis is supported by the fact that ezetimibe in *Asbt*^{-/-} mice did not significantly affect net intestinal cholesterol balance. In *Asbt*^{-/-} mice cholesterol from the intestine is not expected to reach the BBM and therefore blocking the internalization with NPC1L1 process has no relevant additional effect on intestinal and subsequent fecal cholesterol concentrations.

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We showed that *Asbt*^{-/-} mice had a 3-fold increase in fecal BA and NS excretion. While it was previously reported that *Asbt*^{-/-} mice have decreased fractional cholesterol absorption (55% versus 74% in WT littermates) (12), it remained unclear whether this could quantitatively account for the strongly increased fecal NS excretion (17,34). Our data show that fractional cholesterol absorption in *Asbt*^{-/-} mice was much lower (~6%), and even similar to that of WT mice treated with ezetimibe (~4%), suggesting that the increase in fecal NS excretion was mainly due to impaired cholesterol (re)absorption. The differences between the fractional absorption in our experiment and those by Dawson *et al.* could possibly be explained by differences in genetic background (C57BL6 in this experiment versus 129S6/SvEv by Dawson *et al.*), dietary composition (such as different cholesterol and fiber content) or methodology (dual tracer method with blood sampling in this experiment versus dual labeled isotope method with fecal sampling by Dawson *et al.*). Our conclusion that decreased absorption underlies the increase in fecal NS excretion in *Asbt*^{-/-} mice was further supported by the finding that treatment of *Asbt*^{-/-} mice with ezetimibe did not result in an additional increase in fecal NS excretion. Our interpretation of these two observations is that the increase in fecal NS excretion in *Asbt*^{-/-} mice is due to decreased intestinal cholesterol (re)absorption.

Although we were able to profoundly increase fecal sterol output via inactivation of *Asbt* and/or ezetimibe treatment, plasma cholesterol levels and total body cholesterol pool did not change (**Fig S2-S4**). Additionally, bodyweight was similar in WT and *Asbt*^{-/-} mice and unaffected by ezetimibe treatment (data not shown). Maintaining cholesterol homeostasis is essential to the body as it is an important component of cells and precursor for steroid hormones. It has been shown that mice have a great capability to maintain the whole body cholesterol pool size under various conditions (42). However, modulating cholesterol fluxes can impact cholesterol distribution over the different compartments in the body, especially hepatic, plasma and lipoprotein levels. Ezetimibe has been shown in humans to effectively lower LDL-c while maintaining a constant cholesterol body pool size (43). Present observations together with the previously shown effects on hypercholesterolemia in humans and mice show the profound capacity to modulate cholesterol fluxes and lipoprotein distribution without affecting homeostasis essential for sustaining development. Adaptation of the cholesterol *de novo* synthesis rate is a major mechanism to balance the body cholesterol pool size (**Fig 7, Fig S4**). It can therefore be predicted that simultaneous interference with the

cholesterol synthesis capacity will allow more robust manipulations of cholesterol homeostasis, which could contribute to targeted therapeutic strategies.

Ezetimibe did not affect fecal neutral sterol excretion in *Asbt*^{-/-} mice but did augment fecal BA excretion resulting in increased total fecal sterol excretion. *Asbt*^{-/-} mice have a profoundly induced *Cyp7a1* expression (**Fig. S1**), a compensatory reaction to account for fecal BA loss. Therefore, the further augmented cholesterol synthesis by ezetimibe in *Asbt*^{-/-} mice compared to untreated *Asbt*^{-/-} mice (**Fig. 6A**) might be preferentially converted to BAs in the presence of high *Cyp7a1* expression. An elevation in hepatic BA synthesis upon ezetimibe treatment in *Asbt*^{-/-} mice is supported by an increase in fecal BA excretion, which was absent upon ezetimibe treatment in WT mice. Previous studies on the effect of ezetimibe on fecal BA excretion have been conflicting, varying from no change in mice and an increase in humans (19) to no effect in either mice and humans (43). Both ezetimibe and ASBT inhibition have similar benefits on atherosclerosis development in mouse models (44). However, combining both therapies to combat atherosclerosis has to our knowledge not been investigated. While ezetimibe is not able to augment NS excretion further in addition to ASBT inhibition, a combination of these two therapeutic strategies might still be beneficial due to the added increase in cholesterol and subsequent BA synthesis.

Altogether, our results demonstrate that most TICE is counteracted by (re)absorption. Based on these findings we propose a model of net intestinal cholesterol balance that represents the resultant flux of intestinal excretion (or TICE) and (re)absorption and is calculated as [fecal neutral sterol excretion] - [dietary + biliary cholesterol input] (**Fig. 2A**). Additionally, we showed that inactivation of Asbt can be used as another model to potently inhibit intestinal cholesterol absorption. Combining inhibition of intestinal cholesterol (re)absorption with active induction of TICE is expected to further enhance the disposal of cholesterol from the body and is therefore an interesting target for the prevention and treatment of hypercholesterolemia, with or without additional manipulation of *de novo* cholesterol synthesis.

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Acknowledgements

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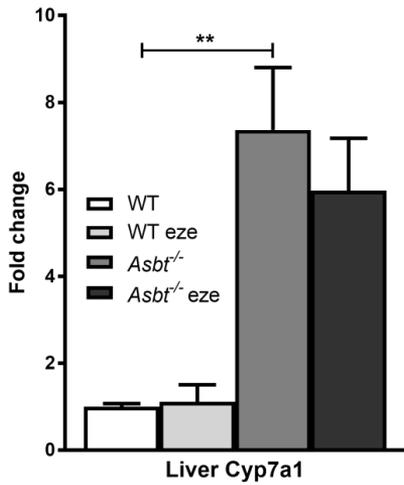6

Supplementary figures

Figure S1. Hepatic Cyp7a1 expression in WT and *Asbt*^{-/-} mice with and without ezetimibe treatment; n=5-6 per group
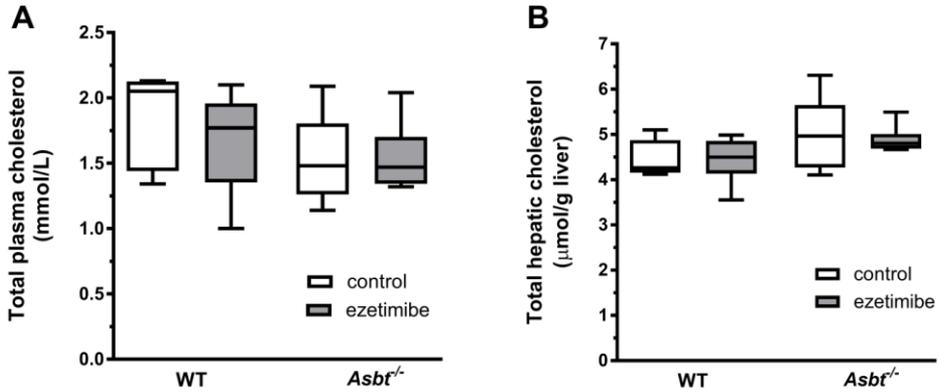

Figure S2. Total plasma and hepatic cholesterol concentrations in WT and *Asbt*^{-/-} mice with and without ezetimibe treatment. Total plasma cholesterol (A) and total liver cholesterol content (B); n=5-6 per group
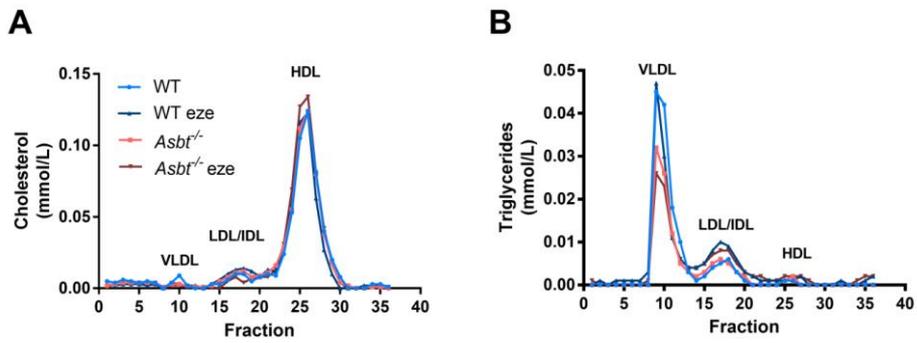
Figure S3. Pooled plasma lipoprotein fractions separated by fast performance liquid chromatography (FPLC). Measured for cholesterol (A) and triglycerides (B); n=5-6 pooled samples per group
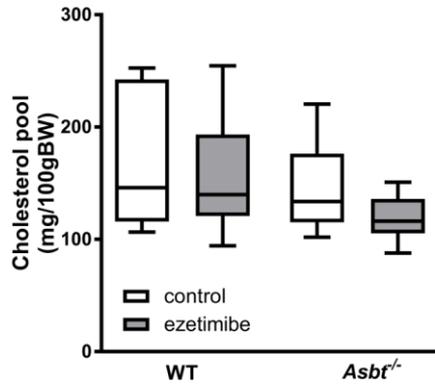

Figure S4. Whole body cholesterol pool in WT and *Asbt*^{-/-} mice with and without ezetimibe treatment. n=5-6 per group
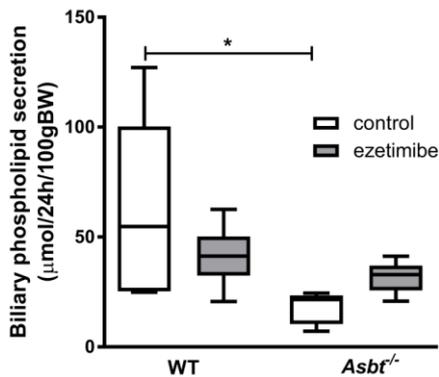

Figure S5. Biliary phospholipid secretion in WT and *Asbt*^{-/-} mice with and without ezetimibe treatment. n=5-6 per group