SUMMARY
Except for conversion to bile salts, there is no major cholesterol degradation pathway in mammals. Efficient excretion from the body is therefore a crucial element in cholesterol homeostasis. Yet, the existence and importance of cholesterol degradation pathways in humans is a matter of debate. We quantified cholesterol fluxes in 15 male volunteers using a cholesterol balance approach. Ten participants repeated the protocol after 4 weeks of treatment with ezetimibe, an inhibitor of intestinal and biliary cholesterol absorption. Under basal conditions, about 65% of daily fecal neutral sterol excretion was bile derived, with the remainder being contributed by direct transintestinal cholesterol excretion (TICE). Surprisingly, ezetimibe induced a 4-fold increase in cholesterol elimination via TICE. Mouse studies revealed that most of ezetimibe-induced TICE flux is mediated by the cholesterol transporter Abcg5/Abcg8. In conclusion, TICE is active in humans and may serve as a novel target to stimulate cholesterol elimination in patients at risk for cardiovascular disease.

INTRODUCTION
The reverse cholesterol transport (RCT) pathway is a crucial antiatherogenic mechanism which mediates the removal of cholesterol from body tissues into feces (Glomset, 1968). The mechanism by which cholesterol is taken up from peripheral cells, including macrophages, has been subject to intense research over the last few decades. It is now generally thought that the ABC transporters ABCA1 (ATP binding cassette subfamily A member 1) and ABCG1 primarily regulate cholesterol efflux from cells, though in the absence of these transporters compensatory pathways may operate. After uptake of the free cholesterol by plasma lipoproteins, the paradigm is that cholesterol is secreted into the intestinal lumen after passage via the hepatobiliary pathway. However, several groups have now demonstrated that direct transintestinal excretion of plasma-derived cholesterol may contribute to RCT (Kruit et al., 2005; van der Velde et al., 2007; Temel et al., 2010; Le May et al., 2013). In mice, this transintestinal cholesterol excretion (TICE) route was shown to account for ~30% of total fecal neutral sterol (FNS) excretion under basal conditions and was enhanced by LiverX-receptor-activated and peroxisome-proliferator-activated receptor-delta (PPAR-δ) agonists (van der Veen et al., 2009; van der Velde et al., 2007; Vrins et al., 2009).

The contribution of TICE to FNS excretion in humans remains, however, to be established. Le May et al. (2013) published the first direct evidence for the activity of the pathway in explants from human small intestine mounted in Ussing chambers. The presence of TICE in humans would offer a plausible explanation for observations reported in early literature. In patients with complete biliary obstruction, a substantial portion of fecal sterols was found to be of nondietary origin (Stanley et al., 1959), and in bile-diverted patients, the intestinal mucosa was found to secrete 250–400 mg of cholesterol per day (Cheng and Stanley, 1959). A human intestinal perfusion study suggested the existence of TICE in humans, with the presence of a substantial flux of cholesterol from enterocytes directly in the intestinal lumen (Simmonds et al., 1967). These and a number of other early reports (Deckelbaum et al., 1977; Hellman et al., 1955; Rosenfeld and Hellman, 1959) were highly criticized, predominantly due to the small number of observations and the study limitations of bile-diverted conditions, including compromised cholesterol absorption and strongly upregulated cholesterol and bile salt synthesis. These studies may have been disregarded due to limitations of intestinal perfusion studies, such as the absence of food; biliary and pancreatic components in the rinsed and perfused intestinal
segments; and the specific composition of the perfusate, which may have influenced the excretory capacity of enterocytes. Although these experiments collectively lend support to the existence of TICE in humans, assessment of TICE’s importance relative to the biliary pathway has remained elusive. This is largely caused by the technical challenges faced in the attempt to reliably estimate this flux in humans, which requires simultaneous assessment of cholesterol absorption, biliary secretion, and—as a final common pathway—FNS excretion. By combining previous experience from validated models in mice (van der Veen et al., 2009) and humans (Harchaoui et al., 2009; Jakulj et al., 2013; Stellaard et al., 1984), we set out to quantify body cholesterol fluxes in a group of mildly hypercholesterolemic, healthy, male subjects using a combined stable isotope method with differentially labeled cholesterol molecules and bile salt tracers. To evaluate the dynamic behavior of the various fluxes, the protocol was later repeated in a subgroup of the participants following treatment with ezetimibe. In this series of experiments, we confirm that hepatobiliary secretion is the major cholesterol export pathway in humans under basal conditions. We also provide in vivo evidence for the activity of TICE in humans and, importantly, show that this cholesterol excretion pathway accounts for most of the cholesterol-mobilizing effect induced by ezetimibe. Studies in mice confirmed these results and suggested that most of the ezetimibe-induced TICE flux flows via the cholesterol-transporting heterodimer Abcg5/Abcg8.

RESULTS AND DISCUSSION

Human Volunteers
Fifteen out of 20 screened subjects met the inclusion and exclusion criteria detailed in the Experimental Procedures section. All completed the first experiment focusing on baseline cholesterol fluxes. Ten out of 15 participants consented to participate in the second experiment after a 28-day treatment period with 10 mg ezetimibe daily (until day 0 of the study schedule displayed in Figure 1A). Five subjects dropped out in the second study because the experiment was fairly intense and time consuming. The second experiment started 6.3 ± 0.9 months after completion of the basal experiment. Baseline characteristics of the 15 initial participants and the ten who completed both study periods at the start of each experiment are shown in Table 1. There was no difference between the first and the second group in any of the parameters measured.

Cholesterol Kinetic and Bile Salt Parameters under Basal Conditions
We then set out to investigate the kinetics of cholesterol disposal from the body. Figures 1B and 1C display average curves of fractional enrichments in plasma and feces of intravenously administered $^{13}$C$_2$-cholesterol. Between days 3 and 5, the tracer enrichments of plasma and feces became identical. Apparently, no de-novo-synthesized cholesterol in the intestine was directly shed into the intestinal lumen. The minor contribution of unabsorbed dietary cholesterol did not seem to influence fecal enrichment. With respect to the intravenously administered $^{13}$C$_2$-cholesterol tracer, the fast initial phase of decrease in tracer enrichment is due to the interplay of tracer equilibration in the fast miscible cholesterol pool as well as efflux of unlabeled cholesterol from the periphery (Figures 1B and 1C). The initial label enrichment after intravenous infusion is somewhat higher in the case of ezetimibe-treated subjects (Figure 1C) due to the lower plasma cholesterol levels. The kinetics of the orally administered D$_7$-cholesterol tracer was somewhat delayed due to intestinal absorption and chylomicron transport via the lymph. After uptake of the chylomicron remnants, the cholesterol is redistributed by the liver in very low density lipoprotein (VLDL), which is transformed to low-density lipoprotein (LDL) in the circulation, and the bulk of the LDL cholesterol then cycles back to the liver. The gradual decay in enrichment after the equilibration phase is mainly caused by activity of the RCT pathway. As expected, there was no difference in kinetics in this phase (days 4–7.5) when comparing the decay of orally versus intravenously administered cholesterol tracers (p = 0.3). Clearly, uptake of D$_7$-cholesterol is much lower in ezetimibe-treated subjects (Figure 1C).

To distinguish RCT via the biliary pathway or TICE, we determined biliary cholesterol secretion. Since direct cannulation of the bile duct in humans is impossible for ethical reasons, we developed an indirect method. Biliary cholesterol secretion is known to be driven by hepatobiliary bile salt secretion under physiological conditions, resulting in a quasilinear relationship between the two parameters (Carey and Mazer, 1984). Because of this direct coupling, measurement of bile salt kinetics and the cholesterol:bile salt ratio in bile can be used to calculate biliary cholesterol secretion, provided that the ratio is similar in different patients. We determined cholate kinetics in the volunteers using a deuterium-labeled cholate tracer technique, as previously described in Stellaard et al. (1984). Cholate pool sizes and the fractional elimination rate of total bile salt from blood ($k_{bs}$) are shown in Table S1. The cholesterol:cholate ratio for each participant was determined by eluting bile absorbed by an Enterotest at T = 24 hr (Supplemental Experimental Procedures). Figures 2A and 2B show the relation between the cholesterol and cholate, as well as the chenodeoxycholate concentration eluted from the Enterotests, with regression lines shown for basal and ezetimibe conditions. In Figure 2A, the slope of the line under the basal condition seems somewhat steeper, though not statistically different from the ezetimibe-treated group (p = 0.49). We therefore chose to combine the data for the basal and ezetimibe conditions, and linear fitting of these data in Figure 2A ($r^2 = 0.8$, p < 0.001) yielded a cholesterol:cholate ratio of 0.23 ± 0.03. Data points for chenodeoxycholate were very similar, and regression lines for basal and ezetimibe conditions fully overlapped. We validated the use of the Enterotest as a measure of the cholesterol:bile salt ratio in a separate experiment carried out in five healthy male volunteers. We harvested duodenal bile both endoscopically and via the Enterotest and showed that the cholesterol:cholate ratios obtained using both methods were comparable (Supplemental Experimental Procedures; Figure S1).

Mean biliary cholesterol secretion was calculated to be 783 ± 69 mg/day under basal conditions in 15 participants.

Cholesterol Fluxes and Sources of FNS under Basal Conditions
Using the calculated biliary cholesterol secretion rates, we analyzed the contribution of the various cholesterol fluxes to
FNS excretion. The fluxes were corrected for cholesterol (re)absorption, with intestinal fractional cholesterol absorption corresponding to the difference between oral and intravenous decay kinetics, as described by Zilversmit (1983). Under basal conditions, cholesterol absorption contributed to 24% ± 11%. Of the daily excreted FNS, 97 ± 6 mg was derived from the diet, and biliary cholesterol secretion accounted for 601 ± 63 mg/day. TICE contributed 197 ± 38 mg/day to FNS.

Ezetimibe-Induced Changes in Cholesterol Fluxes and Sources of FNS

Because of the time elapsed between the first and second experiments, we stopped administration of ezetimibe at the onset of the second experiment to monitor return of the FNS to baseline levels. As shown in Figure 3A, ezetimibe increased FNS about 2-fold, and, after day 3, the amount of FNS gradually decreased to return almost back to baseline levels at day 9. Cumulative FNS excretion was linear up to day 4, and the slope decreased thereafter (Figure 3B). Fecal bile salt excretion increased about 50% with ezetimibe treatment, but this increase did not return to baseline (Figure 3C), resulting in a linear increase in the total amount of fecal bile salts excreted during the course of the experiment (Figure 3D).

Ezetimibe significantly reduced plasma total cholesterol (TC) and LDL cholesterol (LDL-C) by 17.3% ± 8.0% and 25.4% ± 8.1%, respectively (Table 1). Plasma high-density lipoprotein cholesterol (HDL-C) and triglyceride (TG) levels were not significantly affected (Table 1). Equivalent to the basal experiment, cholesterol fluxes were measured and calculated from the fractional enrichment of intravenously administered $^{13}$C$_2$-cholesterol.

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**Figure 1. Scheme of Experiment, A, and Combined Plasma and Feces $^{13}$C$_2$ and D$_7$ Cholesterol Enrichment Curves, B and C**

(A) The abbreviation “t.i.d.” indicates three times a day.
(B) Plasma excursions are depicted for intravenously and orally administered stably labeled $^{13}$C$_2$- and D$_7$-cholesterol, respectively, as well as fecal appearance of $^{13}$C$_2$-cholesterol under basal conditions.
(C) Plasma excursions are depicted for intravenously and orally administered stably labeled $^{13}$C$_2$- and D$_7$-cholesterol, respectively, as well as fecal appearance of $^{13}$C$_2$-cholesterol after 4 weeks of treatment with ezetimibe.
and orally administered D7-cholesterol in plasma during the first 3 days, when the ezetimibe effect on sterol excretion was still optimal (Figure 3A). Note that bile was sampled at t = 24 hr. There was no significant difference between the basal data of these ten subjects compared to the original cohort of 15 (data not shown). Ezetimibe did not affect biliary bile salt content or the biliary cholesterol:cholcholesterol ratio (Figure 2). Bile salt pool size and fractional turnover rate were also not significantly affected by ezetimibe (Supplemental Experimental Procedures). However, ezetimibe increased fecal bile salt excretion from 205 ± 27 to 332 ± 40 mg/day (Figure 4E). Ezetimibe had no significant effect on either dietary cholesterol intake (Figure 4A) or biliary cholesterol secretion, which was calculated to be 769 ± 106 mg/day, and thereby did not deviate from baseline in these subjects (Figure 4B). Ezetimibe reduced intestinal fractional cholesterol absorption by 59% (Figure 4C) and increased FNS from 935 ± 80 to 1,834 ± 84 mg/day (Figure 4D). TC synthesis (bile salts + FNS – CHDiet) increased by 2-fold to a total of 1,950 ± 154 mg/day (Figure 4F, p = 0.001). The ezetimibe-induced increase in FNS output was comparable to that seen in previous reports (Lin et al., 2011; Sudhop et al., 2002, 2009). Because ezetimibe selectively inhibits NPC1L1, the increase in FNS loss has always been attributed to inhibition of cholesterol absorption. We now show that the increase in FNS output is mainly caused by a 4-fold increase in TICE (from 252 ± 46 mg/day to 1,024 ± 114 mg/day, p = 0.001, Figure 4G). Figure S2 depicts the effect of ezetimibe on FNS and TICE in individual subjects. As expected, ezetimibe increased FNS excretion in all subjects, although the response was quite variable. A similar response was observed for TICE rate.

Interestingly, and contrary to our expectation, there was no correlation between the effect of ezetimibe on FNS and the percentage of absorption (data not shown). The target of ezetimibe, NPC1L1, is expressed in the human liver, albeit much less than in the intestine (Züñiga et al., 2008), and genomic variants have been associated with increased risk of gallstone disease (Lauridsen et al., 2015). Therefore, ezetimibe could influence biliary cholesterol secretion. However, in accordance with our data, Wang et al. (2008) and recently Kishikawa et al. (2015) found no effect of ezetimibe on the molar ratio of cholesterol/bile salts in bile, indicating that the role of NPC1L1 in gallstone disease may be more complex.

**Whole-Body Sterol Efflux Strongly Correlates with TICE**

Given the low dietary cholesterol intake recorded in this study, most of the sterols excreted in the feces must be derived from de novo synthesis. Cholesterol synthesis in humans comes mainly from extrahepatic sources; the hepatic contribution has been estimated to be around 10% (Dietschy and Turley, 2002). Consequently, most of the FNS originated in the periphery. Figure S3 shows the values for FNS as a function of either biliary cholesterol secretion (Figure S3A) or TICE (Figure S3B) for the ten individual subjects who completed the whole study. FNS output correlated poorly with biliary cholesterol secretion but quite well with TICE, primarily in the condition with ezetimibe. Under basal conditions, TICE is probably not prominent enough to correlate significantly with FNS.

**Studies in Mice on the Mechanism of Ezetimibe-Induced TICE**

We then set out to investigate the mechanism by which ezetimibe induces TICE. C57BL/6J mice were fed a diet with or without 0.005% ezetimibe for 2 weeks. Similar to the procedure in the human study, cholesterol fluxes were determined using stable-isotope-labeled cholesterol tracers.

Similar to our findings in humans, ezetimibe did not affect dietary intake (Figure 5A), though it slightly increased biliary cholesterol secretion (Figure 5B). As expected, cholesterol absorption was strongly inhibited (Figure 5C), and FNS output was enhanced (7.3 ± 0.3 versus 1.9 ± 0.1 mg/day, Figure 5D) in contrast to the human experiment, fecal bile salt excretion was not affected (Figure 5E), yet total sterol synthesis strongly increased (Figure 5F). Biliary cholesterol secretion increased from 0.8 ± 0.04 to 1.0 ± 0.06 mg/day with ezetimibe treatment. However, this was by far not enough to account for the 5.4 mg/day increase in FNS. Since dietary intake was 1.1 ± 0.05 versus 0.9 ± 0.05 mg/day and cholesterol absorption was almost completely blocked, the amount of FNS that could be attributed to dietary intake and biliary output was about 2 mg/day. Consequently, the remaining 5.3 mg/day of FNS was due to TICE (Figure 5G), plus perhaps a small amount of cholesterol derived from shed enterocytes which we, in a previous study, estimated to be about 15% (i.e., ~0.3 mg/day) under baseline conditions (van der Velde et al., 2007). To ascertain whether the increased flux through the enterocytes could be linked back to increased expression of genes, we performed microarray expression analysis. Except for Stard4, which has been implicated in intracellular cholesterol transport (Iaea et al., 2015), no other known gene potentially linked to cellular cholesterol transport or secretion was upregulated in the small intestine. KEGG pathway analysis demonstrated upregulation of pathways involved in xenobiotic metabolism and a slight upregulation of genes involved in cholesterol synthesis (data not shown). Surprisingly, expression of the cholesterol heterodimer

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**Table 1. Baseline Characteristics**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Basal</th>
<th>Basal</th>
<th>Ezetimibe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male subjects, n</td>
<td>15</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Age, years</td>
<td>62.1 ± 2.9</td>
<td>62.7 ± 2.9</td>
<td>62.7 ± 2.9</td>
</tr>
<tr>
<td>Smoking, n</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.7 ± 0.6</td>
<td>25.8 ± 0.7</td>
<td>25.6 ± 0.7</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>142 ± 3</td>
<td>140 ± 3</td>
<td>143 ± 5</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>85 ± 2</td>
<td>84 ± 2</td>
<td>89 ± 3</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.59 ± 0.17</td>
<td>5.62 ± 0.19</td>
<td>4.74 ± 0.12*</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>3.74 ± 0.13</td>
<td>3.81 ± 0.13</td>
<td>2.87 ± 0.08*</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.32 ± 0.07</td>
<td>1.38 ± 0.07</td>
<td>1.41 ± 0.08</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.01 [0.66 – 2.61]</td>
<td>0.95 [0.66 – 1.39]</td>
<td>0.88 [0.78 – 1.89]</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>5.6 ± 0.1</td>
<td>5.5 ± 0.2</td>
<td>5.5 ± 0.1</td>
</tr>
</tbody>
</table>

Data are presented as means ± SEM. *p ≤ 0.05; as compared to basal conditions (n = 10). As triglyceride data were skewed, data were log-transformed prior to testing; untransformed medians and range are presented.
Abcg5/Abcg8 was downregulated and confirmed by QPCR (−39%, p < 0.01; and −44%, p < 0.01, respectively). Since we have shown in an earlier study that Abcg5/Abcg8 may be a key mediator of TICE, we investigated the role of this heterodimer in ezetimibe-induced TICE flux in Abcg8-deficient mice, which phenocopy Abcg5/g8 double knockout mice. In the absence of Abcg8, dietary cholesterol intake did not change (Figure 5A), and biliary cholesterol secretion decreased but was still slightly stimulated by ezetimibe (Figure 5B). Cholesterol absorption was lower in Abcg8−/− mice and strongly inhibited by ezetimibe (Figure 5C). In the absence of Abcg8, ezetimibe had a much smaller effect on FNS (Figure 5D). In contrast to our human study, ezetimibe did not impact fecal bile acid secretion (Figure 5E). Ezetimibe increased total sterol synthesis (Figure 5F), but the effect was much less pronounced compared to wild-type (WT) mice. The decreased impact of ezetimibe on TC synthesis was due to a decrease in TICE (Figure 5G), which dropped from 5.4 ± 0.3 mg/day in WT to 2.0 ± 0.4 mg/day in the ezetimibe condition, indicating that most of the TICE flux is mediated by Abcg5/g8. Similar to the findings in the human study, there was no correlation between biliary cholesterol secretion and FNS in either WT or Abcg8−/− mice (Figures S3C and S3E). However, FNS correlated highly with TICE both in WT and in Abcg8−/− mice (Figures S3D and S3F). Most of the effect of ezetimibe on FNS excretion could be accounted for by an increase in TICE in mice. The effect was, however, more pronounced in mice, probably because the inhibition of NPC1L1 was stronger. Disruption of gene expression of the heterodimer Abcg5/g8 decreased the effect of ezetimibe on FNS by about 50%. Since ezetimibe is selective for NPC1L1, there must be an interaction between this protein and Abcg5/g8—probably at the level of the brush border membrane. The ABCG5/G8 transporter and NPC1L1 are coexpressed in the small intestine in both humans and mice (Masson et al., 2010). Because these two transporters have opposing functions, continuous cycling of cholesterol at the apical membrane apparently occurs, generating a highly sensitive mechanism to control cholesterol levels in enterocytes. The net fluxes under the different conditions, as obtained in the current study, are given in the simplified scheme of cholesterol transport depicted in Figure 6. Assuming that the inhibition in fluxes measured in the Abcg8−/− mice can be translated directly to the WT controls, we have calculated the flux distribution in absence and presence of ezetimibe. Net influx via NPC1L1 in the absence of ezetimibe is calculated from the dietary intake, biliary cholesterol secretion, and absorption. Net efflux = TICE can be calculated from the stable isotope enrichments, as explained in the Supplemental Experimental Procedures, or can simply be calculated from fecal excretion minus biliary secretion and dietary intake, corrected for absorption. The data show that, in both WT and Abcg8 KO mice, ezetimibe mobilizes cholesterol from the basolateral side of the enterocyte, although we cannot exclude that some of the cholesterol may be synthesized in the enterocyte. The effect is 3-fold higher in WT compared to Abcg8−/− mice.

We speculate that inhibition of NPC1L1 will lead to a higher cholesterol content of the apical membrane of the enterocytes, inducing increased activity of Abcg5/g8; this is in line with a recent study by Nakano et al. (Nakano et al., 2016). Note that ezetimibe increased TICE also in Abcg8−/− mice, indicating that the heterodimer is not the only transporter involved in cholesterol secretion by the enterocytes. Activity of Abcb1a and Abcb1b may be responsible for this phenomenon (Le May et al., 2013). However, we also cannot exclude that ezetimibe induces some cell shedding. In mice, ezetimibe treatment did not affect bile salt excretion, though in humans, we observed significantly increased fecal bile salt output. This is in line with observations in healthy individuals who were sequentially treated with ezetimibe and ezetimibe combined with plant sterols for 3 weeks each as compared to placebo (Lin et al., 2011). Furthermore, a trend toward an increase in fecal BA loss was found in males treated with ezetimibe for 2 weeks (p = 0.068) (Sudhop et al., 2002). Recently, ezetimibe was shown to alter the composition of the microbiota (Catry et al., 2015; Zhong et al., 2015). Although the effect of changes in microbiota on bile salt composition was not reported in these studies, it seems reasonable to speculate that alterations in microbiota composition contributed to the changes in bile salt excretion observed in the present study.

**Study Limitations**

An important step in quantification of TICE in humans in our study is the determination of biliary cholesterol secretion. For ethical
reasons, we were not able to perform direct measurements of biliary cholesterol secretion in humans. Since biliary cholesterol secretion is driven by bile salt secretion, we chose a less invasive bile salt kinetic approach. Bile salt pool sizes were measured according to an established method from our laboratory (Bisschop et al., 2004; Stellaard et al., 1984) and were similar to those in other populations (Northfield and Hofmann, 1975). The product of biliary bile salt secretion and biliary CH:cholate ratio, as measured using the Enterotest, generated the biliary cholesterol secretion. Although the method is based on a number of assumptions, the values for biliary cholesterol secretion in this study are in line with literature data obtained at the time when experimental procedures were less bound to ethical constraints (see Carey and Mazer, 1984, for review). We validated our results from the Enterotest in a separate experiment with five volunteers, in which CH:cholate ratios measured with the Enterotest were similar to those in duodenal sampled bile (Figure S1). It therefore seems reasonable to assume that our estimation of biliary cholesterol secretion is valid. We would like to stress that even though we cannot exclude experimental error in the estimation of biliary cholesterol, in line with results from recent studies (Wang et al., 2008; Kishikawa et al., 2015), ezetimibe treatment does not increase the biliary bile salt:cholesterol ratio. Hence, increased biliary cholesterol secretion can by no means explain the increases in FNS induced by ezetimibe.

TICE accounted for most of the cholesterol mobilized by the drug. Recent specific targeting of intestinal RCT showed profound effects on CVD progression (Lo Sasso et al., 2010) and macrophage RCT (MiGen Investigators et al., 2014; Temel et al., 2010; Yasuda et al., 2010). Our findings imply that stimulation of TICE may explain, in part, the large effects seen on CVD incidence in humans with mutations in the gene encoding NPC1L1 (MiGen Investigators et al., 2014). Given the fact that robust stimulation of biliary cholesterol secretion is not possible in humans due to the high cholesterol saturation index of bile, stimulation of TICE may serve as an alternative strategy to increase cholesterol disposal and reduce the risk of cardiovascular events.

**EXPERIMENTAL PROCEDURES**

**Humans and Animals**

Subjects were recruited via advertisements in local newspapers. Adult males with plasma LDL-C concentrations between 2.8 and 5.0 mmol/L were considered eligible as long as they did not meet the following exclusion criteria: use of medication or plant sterol- or stanol-enriched food products; a history of arterial disease, including unstable angina, myocardial infarction, transient ischemic attack, or cerebrovascular accident; gallstone or other biliary disease; diabetes mellitus; thyroid illness; uncontrolled hypertension; familial hypercholesterolemia (FH), diagnosed either by genotyping or by WHO diagnostic criteria; plasma TG concentrations >3.0 mmol/L; BMI >30 kg/m²; or excessive alcohol consumption. This study was approved by the Institutional Review Board of the Academic Medical Center in Amsterdam. Each participant gave written informed consent.

Male, 12-week-old Abcg8 knockout mice and their C57BL/6J WT littermates (Klett et al., 2004) were housed in a light-controlled (12:12) and temperature-controlled (21°C) facility and received laboratory chow (RMH-B, Hope Farms) **Figure 3. Fecal Neutral Sterol and Bile Salt Excretion before and after Treatment with Ezetimibe**

(A) After stopping ezetimibe treatment at t = 0, fecal neutral sterol (FNS) gradually decreased toward baseline levels (n = 10).

(B) Total FNS production in time under basal and after 4 weeks of ezetimibe treatment (n = 10).

(C) Ezetimibe increases fecal bile salt output (n = 10).

(D) Fecal bile salt output increases linearly in time (n = 10).
Figure 4. Cholesterol Fluxes under Basal and Ezetimibe-Treated Conditions in Humans

(A) Dietary cholesterol intake is depicted.
(B) Biliary cholesterol secretion, calculated as described in the text and Supplemental Experimental Procedures, is depicted.
(C) Fractional cholesterol absorption is depicted.
(D) FNS excretion is depicted.
(E) Bile salt (BS) excretion is depicted.
(F) Total sterol (neutral sterol + bile salt) synthesis is depicted.
(G) Transintestinal cholesterol excretion is depicted.

n = 10; *p < 0.05; **p < 0.01.
ad libitum. All experiments were approved by the Ethics Committee for Animal Experiments of the University of Groningen.

**Human Subjects**

The tracer administration and sampling protocol (Figure 1), based on our prior study in mice (van der Veen et al., 2009), was modified for applicability in humans. Participants kept a cholesterol-restricted diet (<300 mg/day) from 7 days prior to the dual cholesterol tracer administration (T = 0 hr) until the end of study (T = 168 hr). During the study period following T = 0 hr, subjects consumed two supervised meals per day at the research facility and kept a dietary record while maintaining their usual patterns of smoking and physical activity. In order to normalize fecal isotope recovery measurements for variations in fecal flow, subjects ingested tracer capsules containing 3 mg [5,6,22,23-D4]-sitostanol (Cambridge Isotope Laboratories) three times daily with their meals from T = 0 hr until the end of the study. The next morning (T = 0 hr), participants were admitted to the hospital in a fasting state for a 30 min infusion of 50 mg [3,4-13C2]-cholesterol (Isotec) dissolved in 33 mL 10% Liposyn III (Hospira Inc.). The 13C2-cholesterol tracer solution was prepared for injection by dissolving the tracer in warm USP ethanol into a clear solution under sterile conditions. Thereafter, the isotope and ethanol mixture was added to 10% Liposyn III for a total administrable volume of 33 mL. Immediately after infusion of the intravenous tracer dose, subjects ingested 50 mg of [25,26,26,26,27,27,27-D7]-cholesterol (Cambridge Isotope Laboratories), administered in a stomach-soluble gelatine capsule, together with a standardized breakfast, as previously described (Jakulj et al., 2013). Subjects refrained from eating for 4 hr following the test meal to allow complete gastric emptying. Blood samples were collected directly before the 13C2-cholesterol infusion and at 4, 9, 11, 23, 25, 28, 30, 48, 72, 120, and 168 hr after administration of the cholesterol tracers. Plasma was isolated by centrifugation and stored at −80°C for subsequent cholesterol and bile salt extraction and measurement of the tracer enrichments, as described below. Subjects collected daily fecal samples from T = 0 hr until the end of study (T = 168 hr). At T = 24 hr, a biliary sample was obtained in the morning fasting state by use of the Enterotest (HDC Corp). This commercially available device for sampling of gastrointestinal contents was used to determine biliary bile salt and cholesterol contents, as previously described (Muraca et al., 1991; Van der Meer et al., 1990). Participants swallowed the encapsulated nylon thread with water in the evening before the T = 24 hr study visit; one end was taped at a corner of the mouth. The capsule dissolves in the stomach, and the thread, weighed at its distal end, passes into the duodenum. The next morning (T = 23 hr), an intravenous dose of 0.05 mg/kg synthetic cholecystokinin (CCK) (sincalide, Kinevac, Bracco Diagnostics) was administered to induce gallbladder contraction. After 1 hr, the thread, which was colored yellow with bile at the distal end, was withdrawn and immediately frozen at −80°C for subsequent cholesterol and bile salt extraction and measurement of tracer enrichments. Finally, during a period of 5 hr following the CCK dose, plasma was obtained at 30 min intervals for measurement of bile salt profiles in order to estimate bile salt turnover as described in the Supplemental Experimental Procedures.

**Figure 5. Cholesterol Fluxes under Basal and Ezetimibe-Treated Conditions in WT and Abcg8−/− Mice**

(A) Dietary cholesterol intake is depicted.
(B) Biliary cholesterol is depicted.
(C) Fractional cholesterol absorption is depicted.
(D) FNS excretion is depicted.
(E) Bile salt (BS) excretion is depicted.
(F) Total sterol (neutral sterol + bile salt) synthesis is depicted.
(G) Transintestinal cholesterol excretion is depicted.

n = 8–10; *p < 0.05; †p < 0.01.

(D2-CA, Sigma Aldrich, Isotec Inc.) dissolved in 200 mL 0.5% sodium bicarbonate in water together with 0.4% dextrose. The cholate tracer was used to measure bile salt pool size and turnover rate, based on measurement of enrichment in postprandial plasma in the subsequent 4 days (Stellaard et al., 1984).
A second experiment was performed according to the same tracer administration and sampling protocol after subjects had used 10 mg ezetimibe daily for a period of 28 days before T = 0.

Mice
To assess fractional cholesterol absorption and cholesterol kinetics, mice received an intravenous dose of 0.3 mg (0.73 μmol) cholesterol-D5 (Medical Isotopes, Inc.) dissolved in Intralipid (20%, Fresenius Kabi) and an oral dose of 0.6 mg (1.535 μmol) cholesterol-D7 (Cambridge Isotope Laboratories, Inc.) dissolved in medium-chain TG oil (Pharmacy UMCG) 10 days prior to the end of the experiment. Blood spots were collected from the tail at 0, 3, 6, and 12 hr and subsequently every 24 hr after administration of the stably labeled cholesterol. At the end of the experiment, mice were anesthetized by intraperitoneal injection with Hypnorm (1 mL/kg; Janssen Pharmaceuticals) and Diazepam (10 mg/kg; Actavis). After ligation of the bile duct, the gallbladder was cannulated. Bile collected during the initial 5 min after cannulation was disposed to ensure no gallbladder bile was collected; subsequently, hepatic bile was collected for 30 min (Kuipers et al., 1996). During the collection period, body temperature was stabilized in a humidified incubator. After bile had been collected, mice were sacrificed by cardiac puncture and tissues were rapidly excised.

Analytical Procedures
Fasted plasma TC, HDL-C, and TG concentrations were measured with standard automated methods; LDL-C concentrations were calculated using the Friedewald formula (Friedewald et al., 1972).

Plasma cholesterol was extracted with ethanol and acetone (1:1) (Neese et al., 1993) and derivatized using N,O-bis-(trimethylsilyl)trifluoroacetamide (1% trimethylchlorosilane) and pyridine (1:1) at room temperature. Bile salts were deconjugated, extracted from plasma, and derivatized according to Hulzebos et al. (2001).

Biliary lipids were extracted according to Bligh and Dyer (1959) from the freshly frozen bile samples and from bile eluted from the Enterotests as previously described (Muraca et al., 1991). Biliary free cholesterol was derivatized using N,O-bis-(trimethylsilyl)trifluoroacetamide and pyridine (1:1) with 1% trimethylchlorosilane at room temperature.

Daily frozen fecal samples were thawed and homogenized with distilled water (1:1; w/w). A total of 10 mL of fecal homogenate was dispensed into a 10 mL plastic tube. Bile salts were extracted and quantified as their methyltrimethylsilyl derivatives according to Setchell et al. (1983). Bile salts were analyzed by gas chromatography (Agilent 6890) using a CPSil 19 capillary column (25 m × 0.25 mm × 0.2 μm; Chrompack).

Daily FNS excretion rates were calculated by expressing the results relative to Δ4-sitostanol, measured by gas chromatography/mass spectrometry (GC/MS), as described below. Enrichments of stable isotopes in the FNS were measured in the cholesterol fraction, whereas total FNS was determined as the sum of fecal cholesterol and its bacterial metabolites, which were assumed to have similar specific enrichments to cholesterol.

Plasma and biliary bile salt profiles were determined using liquid chromatography/mass spectrometry (LC-MS/MS) (Api3000; AB Sciex) coupled to a Shimadzu HPLC. The system was controlled by Analyst 1.6 software (AB Sciex). The LC conditions were as follows: an XBridge Shield RP18 column.

Figure 6. Schematic Representation of Cholesterol Transport Pathways through the Enterocyte in WT and Abcg8+/− Mice in Presence and Absence of Ezetimibe
(A) Representation of WT mice.
(B) Representation of Abcg8−/− mice.
Data for the different fluxes (J) were extracted from the experimental data shown in Figure 5. Values for J_{Abcg8+/−} in WT mice were estimated by subtracting FNS in Abcg8−/− mice from FNS in WT mice. In the steady state, J_{NPC1-l1} must be equivalent to the amount of cholesterol absorbed (J_{NPC1-l1}). Data for the condition plus ezetimibe are presented in italics. All data are given as mg/day for 8–10 mice.

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elimination rate of total bile salts from blood (kBS). The latter was estimated using a ZB-5MS capillary column (30 m × 0.25 mm × 0.25 μm; Phenomenex). Isotopic enrichments determined as atom percent excess (APE) were converted into molar percent excess (MPE) using a linear calibration curve, as described by Lee et al. (1991), in order to obtain excess fractional distribution of mass isotopomers M₀–M₇ resulting from isotope dilution. In this approach, M₇ represented the orally administered label.

Isotopic enrichments of 13C₂-cholesterol were measured using gas chromatography combustion-isotope-ratio mass spectrometry (GC-C-IRMS) (Delta Plus Thermo Electron) using a ZB-5MS capillary column (30 m × 0.25 mm × 0.25 μm; Phenomenex). Isotopic enrichments determined as atom percent excess (APE) were converted into molar percent excess (MPE) using a linear calibration curve. D₃-cholate (D₃-CA) was measured on an Agilent 7890A GC connected to an Agilent 5975 MSD (Agilent). Gas-liquid chromatographic separation was performed on a 10 m × 0.100 mm column with a film thickness of 0.1 μm (DB-5MS; Agilent). Isotope ratios were determined in the selected ion monitoring mode on m/z 623.4 (M₀) and 627.3 (M₄).

D₄-sitostanol derivatives were analyzed by GC/MS, electron impact (EI) mode (Agilent 7890A/5975C) using a ZB-5MS capillary column (30 m × 0.25 mm × 0.25 μm; Phenomenex). Ions with m/z 488–493 generated in the electron impact mode corresponding to the m₀–m₅ mass isotopomers were corrected for the fractional distribution due to natural abundance, as described by Lee et al. (1991), in order to obtain excess fractional distribution of mass isotopomers M₀–M₅.

Calculation of Kinetic Parameters and Cholesterol Fluxes
The methods to calculate fractional and absolute contributions of the distinct fluxes to total FNS loss resemble those previously described in mice (van der Veen et al., 2009). From plasma and fecal decay curves of the intravenously administered 13C₂-cholesterol and orally administered D₇-cholesterol tracers, combined with both enrichments in the bile sample, several kinetic parameters were calculated. This was done by use of a mathematical kinetic model (SAAM-II software, The Epsilon Group, version 2.3). Combined with measurement of dietary cholesterol intake, fractional cholesterol absorption, excreted fecal NS and bile salt mass, bile salt pool size, secretion rate, and biliary CH/BS, specific sources of cholesterol contributing to total FNS mass could be calculated as described below. A more detailed description of calculations with justification of equations is provided in the Supplemental Experimental Procedures.

Dietary Intake, Fractional Cholesterol Absorption, and Cholesterol Synthesis
Dietary cholesterol intakes were calculated using a freely available Dutch nutrition database program (www.dietinricht.nl). Fractional cholesterol absorption (Fₐ) was calculated from the area under the curve of intravenously and orally administered cholesterol isotopes and their dose, as described in the Supplemental Experimental Procedures.

Cholesterol synthesis was estimated according to the cholesterol balance method, i.e., as the difference between fecal elimination of cholesterol, both as BS and FNS, and accurately assessed dietary cholesterol intake (Grundy and Ahrons, 1969; Samuel and McNamara, 1993).

B₈⁶S results from the product of bile salt pool size (QBS) and the fractional elimination rate of total bile salts from blood (kBS). The latter was estimated from the plasma decay curves of total bile salt concentrations measured at 30 min intervals during 5 h following administration of the intravenous dose of cholecystokinin (Supplemental Experimental Procedures; Figure S3). Subsequently, B₈⁶S was multiplied by the biliary CH/BS, yielding the biliary cholesterol secretion rate.

Statistical Analyses
Differences in measured fluxes between the basal and repeated experiment after ezetimibe treatment were statistically analyzed by use of paired sample t tests and, if data were skewed, the Wilcoxon signed rank test (SPSS Inc., version 15.0). Values in Figures 4 and 5 are presented as Tukey boxplots. Significance was tested using the Kruskal-Wallis H test followed by Conover posthoc comparisons. *p < 0.05, **p < 0.01. Data in the text are presented as mean ± SE; p < 0.05 was considered statistically significant.

ACCESSION NUMBERS
The accession number for the microarray data is Genome Spatial Events: GSE77236.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.10.001.

AUTHOR CONTRIBUTIONS

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.

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