Transintestinal cholesterol excretion in humans

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Purpose of review
To discuss recent insights into the measurement and cellular basis of transintestinal cholesterol excretion (TICE) in humans and to explore TICE as a therapeutic target for increasing reverse cholesterol transport.

Recent findings
TICE is the net effect of cholesterol excretion by the enterocyte into the intestinal lumen and is the balance between input and output fluxes through the enterocytes. These fluxes are: cholesterol excretion into the intestinal lumen mainly via ATP-binding cassette (ABC) G5/8, cholesterol absorption from the intestine by Niemann-Pick C1 like protein 1, the uptake of plasma lipoproteins by enterocytes at the basolateral membrane, and the excretion of cholesterol in chylomicrons into the lymph. Multiple studies have shown that TICE contributes to fecal neutral sterol (FNS) excretion in humans. TICE can be targeted with plant sterols, liver X receptor agonists, bile acids, ezetimibe, and proprotein convertase subtilisin/kexin type 9 inhibitors.

Summary
TICE contributes significantly to FNS excretion in humans, independently of the biliary pathway. Knowledge about its underlying cellular mechanisms surges through in-vivo and in-vitro studies in mice and humans. TICE might be an interesting therapeutic target for increasing cholesterol disposal with the feces. Albeit multiple therapeutic options are available, studies showing clinical benefit are still needed.

Keywords
ATP-binding cassette G5/8, ezetimibe, Niemann-Pick C1 like protein 1, transintestinal cholesterol excretion

INTRODUCTION
Cholesterol is essential for life in humans and most animals. It serves multiple functions in the human body as it is a crucial part of cell membranes and serves as a precursor for bile acids, steroid hormones, and vitamin D. Cholesterol metabolism is tightly regulated, with a major role for the liver and intestine. Oral intake serves as an essential source of cholesterol; however, most cells are capable of cholesterol synthesis itself, explaining the modest effects of altering dietary cholesterol intake on plasma cholesterol levels [1]. Unfortunately, an excess of cholesterol (i.e., hypercholesterolemia) can accumulate in the arterial wall and leads to the development of atherosclerotic cardiovascular diseases (CVD), the cause of ~25% of deaths globally [2]. This process is predominantly caused by the uptake of cholesterol-rich LDLs particles by arterial wall macrophages and consequently the formation of foam cells, leading to atherosclerosis [3,4].

As cholesterol cannot be catabolized to a major extent within the human body except for conversion to bile acids, the reverse cholesterol transport (RCT) pathway is an essential antiatherogenic tool by facilitating the removal of excess cholesterol from the body via fecal excretion [5]. RCT is generally defined as the efflux of cholesterol from peripheral tissues (e.g., atherosclerotic plaques), transportation to the liver by HDLs, and subsequent hepatobiliary secretion into the feces. Nowadays, a second RCT pathway is known to contribute to fecal excretion of cholesterol: the direct transintestinal cholesterol excretion (TICE) of plasma-derived cholesterol by enterocytes into the lumen of the small intestine. There is strong evidence of the existence of TICE in mice and rats [6–9] and accumulating evidence of existence of TICE in humans.
**KEY POINTS**

- TICE is present in humans and contributes significantly to fecal sterol neutral excretion.
- Measuring TICE in humans requires the use of stable isotopes to measure cholesterol fluxes.
- ABCG5/8 and ABCB1a/b promote cholesterol excretion into the intestinal lumen.
- The effect of ezetimibe on FNS excretion in humans is mainly due to increased TICE.
- ABCG5/8-dependent cholesterol excretion can be increased by plant sterols, bile acids, and liver X receptor agonists.

The purpose of this review is to discuss the recent insights into the measurement and molecular mechanism of TICE in humans. Furthermore, TICE’s potential as a therapeutic target for the removal of excess cholesterol from the human body will be discussed.

**QUANTITATION OF TRANSINTESTINAL CHOLESTEROL EXCRETION**

Four cholesterol fluxes contribute to TICE: cholesterol excretion into the intestinal lumen, cholesterol absorption from the intestine into enterocytes, the uptake of plasma lipoproteins by enterocytes at the basolateral membrane, and the excretion of cholesterol, together with triglycerides, packed in chylomicrons into the lymph. Under in-vivo conditions, TICE flux cannot be determined directly and has to be calculated from fecal neutral sterol (FNS) excretion minus the contribution of biliary and dietary input. Subsequently, both fluxes have to be corrected for intestinal absorption. Van der Velde et al. circumvented this procedure by quantifying TICE directly through performing perfusion experiments in selected parts of the small intestine in mice [6]. This procedure successfully assessed TICE activity and demonstrated the strict dependency of TICE on a luminal cholesterol acceptor. The advantage of this method is the possibility to study the mechanism of TICE in a direct way. The disadvantage is that normal intestinal physiology (motility) is disrupted because of the surgery and for this reason it cannot be performed in humans. Calculation of TICE under in-vivo conditions is relatively straightforward in mice. Dietary intake can be measured accurately and biliary cholesterol secretion can be estimated by collecting bile. Using a dual (stable) isotope method, cholesterol absorption can be quantified, making calculation of TICE possible. A disadvantage of this relatively simple approach is that it does not take into account the cholesterol synthesized in the enterocytes and cholesterol directly excreted or shedded from dead cells. Computational modeling of the isotope decay curves can address this problem and successfully quantify the flux from the blood into the intestinal lumen. Jakulj et al. [11**] adapted this approach for the use in humans. The cellular mechanisms, proteins, and transporters driving the fluxes present in TICE will be discussed below.

**TRANSINTESTINAL CHOLESTEROL EXCRETION AT THE CELLULAR LEVEL**

**Intestinal cholesterol excretion**

The ATP-binding cassette (ABC) G5 and G8 (ABCG5/8) heterodimer acts as efflux transporter at the apical membrane of the enterocyte and hepatocyte where they promote secretion of cholesterol and plant sterols [13]. After synthesis in the endoplasmic reticulum, ABCG5 and ABCG8 dimerize, followed by transport to the apical plasma membrane [13–15]. Dimerization is essential because the individual proteins do not reach the apical membrane. The importance of ABCG5/8 in TICE surfaced in a murine study demonstrating that on the one hand liver X receptor (LXR) activation increases both TICE and ABCG5/8 expression, whereas TICE is decreased in mice lacking ABCG5 [16]. Intestinal ABCG5/8, in mice lacking hepatic ABCG5/8, is capable to excrete cholesterol into the intestinal lumen, implicating that intestinal ABCG5/8 contributes to nonbiliary cholesterol excretion [17**]. Surprisingly, intestinal perfusion studies failed to detect the stimulating activity of ABCG5/G8 seen in in-vivo experiments. Apparently, a stimulating factor is missing in the perfusate which is present in vivo [6].

TICE cannot be entirely attributed to the ABCG5/8 heterodimer as a significant amount of TICE is still active in mice lacking ABCG5 or ABCG8, suggesting that other apical cholesterol transporters are involved in TICE as well [16,18,19**]. Le May et al. [10*] suggested that the multidrug transporter ABCB1a/b could be involved. This protein is expressed at the apical side of enterocyte and may act as a cholesterol transporter [20]. It was demonstrated in mice lacking ABCB1a/b that TICE was decreased by 26.5% and resulted in decreased FNS excretion. Using a selective pharmacological ABCB1a/b inhibitor, TICE was significantly reduced in wild-type mice and not in the knockout mice, indicating the involvement of ABCB1a/b in TICE.
Thus, an interplay of the apical transporters ABCG5/8 and ABCB1a/b in the enterocyte may be responsible for TICE activity in mice. However, it would be interesting to investigate whether TICE is still active in mice lacking ABCG5/8 and ABCB1a/b to ascertain if these transporters are the only apical transports involved in intestinal cholesterol excretion in TICE or that other mechanisms are also involved, and how these findings can be translated to humans.

**Intestinal cholesterol absorption**

The major pathway by which dietary and biliary cholesterol are taken up by enterocytes is through the Niemann-Pick C1 like protein 1 (NPC1L1; Fig. 1). NPC1L1 knockout mice showed a 64–69% reduction in cholesterol absorption from the intestinal lumen [21,22]. The same effect is accomplished by blocking NPC1L1 with ezetimibe, a drug now widely used for LDL cholesterol (LDL-C) lowering in hypercholesteremic patients. Rodents receiving ezetimibe showed a 92–96% reduction of cholesterol absorption [23]. The discrepancy in reduction of cholesterol absorption between NPC1L1 knockout mice and inhibition with ezetimibe has still to be elucidated, but one may speculate that the lack of NPC1L1 transporters in knockout mice is compensated through another transporter. Treatment of humans with ezetimibe monotherapy results in less intestinal cholesterol absorption and lowers LDL-C plasma levels by 15–20%, despite an 89% increase in endogenous cholesterol synthesis [24]. The cholesterol absorption inhibiting effects of ezetimibe are

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**FIGURE 1.** Cellular transporters and cholesterol fluxes involved in transintestinal cholesterol excretion. At the apical side of the enterocyte, dietary and biliary cholesterol are absorbed from the intestinal lumen by Niemann-Pick C1 like protein 1, whereas intracellular cholesterol is excreted by ATP-binding cassette G5 and G8, ATP-binding cassette B1 a and b, and possibly other unidentified mechanisms. Moreover, intracellular cholesterol is excreted in chylomicrons into the lymph at the basolateral side. Plasma cholesterol is derived from lipoprotein particles, possibly through endocytoses with the LDL receptor or other mechanisms. ABCB1a/b, ATP-binding cassette B1 a and b; ABCG5/8, ATP-binding cassette G5 and G8; NPC1L1, Niemann-Pick C1 like protein 1; LDLR, LDL receptor; VLDL, very LDL; PCSK9, proprotein convertase subtilisin/kexin type 9.
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nowadays well established in mice and humans. Interestingly, studies in mice have shown that ezetimibe not only affects cholesterol absorption in the intestinal lumen but also stimulates cholesterol excretion from enterocytes through the ABCG5/G8 heterodimer [25]. Nakano et al. [26] investigated this process in more detail and proposed a model in which cholesterol content in the brush border membrane (BBM) of the enterocytes is the driving force for NPC1L1-dependent cholesterol uptake. An increased cholesterol efflux from the BBM to the intestinal lumen was seen when NPC1L1 was inhibited with ezetimibe in mice. Moreover, it was shown that this efflux was higher in wild-type mice treated with ezetimibe than in ABCG5/8 knockout mice, suggesting a promoting role for ABCG5/8 in ezetimibe-induced cholesterol excretion [26]. Albeit, the exact mechanism by which NPC1L1 transports cholesterol to the intracellular space is yet not clear, NPC1L1-endocytosis mediated by Numb has been proposed as the underlying mechanism. The absence of Numb, a clathrin-adaptor protein, in vitro and in NPC1L1 knockout mice impaired cholesterol absorption [27]. However, later reports challenged the concept that cholesterol absorption is achieved by endocytosis [28]. Future research should address those contradicting findings.

Basolateral cholesterol uptake by enterocytes

Cholesterol excreted via TICE can originate from different sources: a) intestinal uptake of dietary and biliary cholesterol, b) de-novo synthesis in enterocytes, and c) uptake from the blood. Cholesterol balance studies in humans and mice indicate that when TICE is stimulated by pharmacological means, most of the cholesterol is derived from the blood [9,15]. The pathway via which cholesterol enters the enterocytes is a matter of controversy. As TICE may play an important role in the RCT pathway, uptake from HDL seemed an attractive option. Vrins et al. investigated this mechanism by following the fate of radiolabeled cholesterol incorporated in HDL in Abca1/Sr-b1 double knockout mice. These mice lack endogenous HDL and cannot clear HDL via the liver. No transport of cholesterol into the intestinal lumen could be discerned in these experiments suggesting that HDL could not be the carrier for TICE cholesterol [29]. In contrast, Le May et al. [10] carried out experiments with intestinal explants embedded in Ussing chambers and could demonstrate cholesterol transport across the intestinal tissue from HDL as well as LDL. The reason for the discrepancy between the data of Vrins et al. and Le May et al. is not clear. Le May et al. reported conflicting data regarding the role of the LDL receptor in mediating TICE flux. They could not find decreased TICE in LDL receptor knockout mice, a result confirmed by both Temel and Brown [30] and our group (unpublished observations). Despite this seemingly lack of evidence for a role of the LDL receptor-mediated pathway, Le May et al. reported that absence of the LDL-receptor modulating protein PSCK9 increased TICE and conversely intravenous injection of this protein in mice inhibited TICE. Taken together, it cannot be excluded that LDL receptor knockout mice compensate for the lack of this receptor and upregulate another receptor, or proprotein convertase subtilisin/kexin type 9 (PCSK9) blocks an alternative route of cholesterol import. When indeed both LDL and HDL can be excluded as donors of TICE cholesterol, VLDL seems an attractive alternative candidate. Marshall et al. tested this hypothesis by treating mice with antisense oligonucleotides targeting microsomal transfer protein. This treatment strongly inhibited VLDL secretion and FNS excretion, particularly in liver-specific transgenic NPC1L1 overexpressing mice. As these mice do not show biliary cholesterol secretion, the effect on the FNS must have been due to a decrease in TICE. The authors conclude that, at least, VLDL secretion is required to feed this pathway with cholesterol [31].

Transintestinal cholesterol excretion in humans

In 1959, two studies observed that in patients with complete biliary obstruction, the intestinal mucosa still excreted 250–400 mg cholesterol per day indicating that an alternative route of cholesterol excretion exists in humans [32,33]. Under more physiological conditions, Simmonds et al., using intestinal perfusion of the jejunum in humans, estimated that ~44% of FNS originated from non-biliary pathways [34]. In 2013, new evidence for activity of TICE in humans was provided by Le May et al. [10*] using isolated intestinal explants in Ussing chambers as described above. The existence of TICE in humans was further established recently with the publication of Jakulj et al. [11**]. Under basal conditions, TICE contributed for 35% of FNS excretion in 15 men with mild hypercholesterolemia, when using stable isotope-based technology as described in this review. Subsequently, 10 patients were treated with ezetimibe 10 mg/day, resulting in a significant four-fold increase in TICE (from 252 ± 46 to 1024 ± 114 mg/day). Those results underline the TICE mechanisms in mice to be present in humans as well. Dugardin et al. [12] studied transepithelial cholesterol transport in the human Caco-2/TC7 cell line to demonstrate that
differentiated Caco-2T7 enterocyte cells take up cholesterol at the basolateral membrane and then excrete the cholesterol through the apical membrane. In line with earlier findings of van der Velde et al. [6], they found that phosphatidylcholine/taurocholate micelles modify the intracellular distribution of cholesterol and facilitate its transport from subbasolateral to subapical areas of the enterocyte. Furthermore, it was demonstrated that less TICE occurred when the cellular microtubules were disrupted with colchicine and nocodazole, suggesting that the microtubule cytoskeleton is important for intracellular cholesterol transport.

Transintestinal cholesterol excretion as a therapeutic target

As highlighted in this review, the TICE pathway is a major nonbiliary contributor to FNS excretion and RCT in mice and humans. Compared with the stimulation of the hepatobiliary cholesterol excretion, which may promote gallstone formation [35], increasing intestinal cholesterol excretion by the TICE pathway is an attractive approach to remove the excess of cholesterol and prevent atherosclerosis. See Figure 2 for an overview of the therapeutic targets discussed below. Recent research has shown that manipulation of the cycling of cholesterol between NPC1L1 and ABCG5/8 seems the most attractive option to stimulate TICE. Inhibition of NPC1L1 by ezetimibe is straightforward and stimulates TICE, whereas simultaneous activation of ABCG5/8 possible enhances TICE even more.

LXR regulates the expression of hepatic and intestinal ABGG5/8 and could therefore be an interesting pharmacological target for TICE [16,36–38]. Studies have shown that activation of LXR, through cholesterol feeding, dietary plant sterols, or agonist administration increases hepatic and intestinal ABCG5/8 expression [18,36,38] as well as biliary cholesterol concentrations in mice [36], whereas these effects were absent in mice lacking ABCG5/8 [37]. Moreover, murine studies demonstrated that TICE is directly stimulated with LXR agonists and that FNS excretion upon LXR activation is independent of hepatobiliary sterol secretion in mice [7,16]. To this end, administering a LXR agonist to increase intestinal ABCG5/8 expression in humans could be an interesting approach for targeting TICE in humans. However, an intestinal-specific agonist for LXR-induced TICE should be used to avoid hepatic side effects, such as increased hepatic lipogenesis, steatosis, and hypertriglyceridemia [39,40]. Several first-phase clinical trials with LXR agonists were performed with beneficial effects regarding RCT. However, one study had to be terminated early because of unexpected adverse neurological events [41], whereas other trials do not disclose the reasons for early termination and were not published [42]. Adding plant sterols to the diet has similar effects to LXR agonists. Plant sterols intake lowers total cholesterol and LDL-C in normolipidemic and hypercholesterolemic patients without affecting HDL-cholesterol [43], via competition with cholesterol for incorporation in mixed micelles. This leads to a decreased fractional cholesterol absorption and increased fecal cholesterol excretion [44]. Plant sterols may be able to activate LXR followed by the upregulation of ABCB1 and ABCG5 [45–47]. Brufau et al. investigated whether plant sterols feeding stimulates FNS via TICE on an ABCG5/8-dependent manner. They found that plant sterols feeding increased FNS in mice mainly by an increase in TICE and that ABCG5/8 plays a nonexclusive role in the preservation of TICE [18].

Recently, de Boer et al. [19**] reported that TICE is also regulated by intestinal FXR via induction of its target gene FGF15 in mice and FGF19 in rats and humans. Bile acids are ligands for FXR and binding results in the release of FGF19 from the small intestine into the circulation, which contributes to regulation of bile acid synthesis and postprandial metabolism [48,49]. Stimulation of the FXR-FGF15 pathway with an FXR agonist and ezetimibe in mice led to an estimated excretion of 60% of their total body pool of cholesterol content each day [19**]. Moreover, administration of a FXR agonist or FGF19 induced the muricholate: cholate ratio in bile, creating a more hydrophilic bile acid pool, resulting in secretion of cholesterol into the intestinal lumen via ABCG5/8. Notably, the increase in TICE induced by the FXR agonist was independent of changes in cholesterol absorption via NPC1L1. Thus, a therapy that combines the effect of FGF19 induction on ABCG5/8 by bile acids and simultaneous inhibition of intestinal cholesterol absorption by ezetimibe would supposedly lead to an increase in TICE. Wang et al. [17**] found that ursodiol increases hepatic ABCG5/8, biliary secretion, and FNS elimination and acts additively to ezetimibe to increase FNS excretion in mice. Remarkably, the combination of ursodiol and ezetimibe also increased FNS excretion in ABCG5/8-deficient mice, leading to the conclusion that this combination increases ABCG5/8 expression and FNS excretion, but the latter effect was ABCG5/8 independent.

Another therapeutic modulator of TICE might be PCSK9, an interesting target in the light that PCSK9 inhibition is more and more prescribed as an LDL-C-lowering agent for the prevention of CVD. The PCSK9 protein binds the LDL receptor and enhances its intracellular lysosomal degradation,
resulting in an impaired LDL-C uptake in the liver. Although the role of the LDL receptor in TICE is still unclear, PCSK9 has been shown to modulate TICE [10*,12]. When recombinant PCSK9 is added to Caco-2/TC7 cells at the basolateral compartment, transcellular cholesterol transport is decreased by 28% [12]. PCSK9 knockout mice showed increased TICE, which could be blocked by an injection of recombinant PCSK9 [10*]. Data in humans is still lacking, but it can be hypothesized that PCSK9 inhibition with the now available antibodies might increase FNS in addition to its plasma LDL-C-lowering effect via the liver.

**CONCLUSION**

TICE is the net effect of cholesterol excretion by the intestine exclusively and serves as a second, non-biliary, RCT pathway in humans. The rate of TICE depends on the balance between four fluxes; intestinal cholesterol excretion through ABCG5/8 and ABCB1a/b, cholesterol absorption through NPC1L1,
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cholesterol uptake and cholesterol excretion at the basolateral membrane of enterocytes. Targeting TICE to enhance RCT might be an attractive target to attenuate CVD risk and might be accomplished with plant sterols, LXR agonists, bile acids, ezetimibe, and PCSK9 inhibitors. Exciting times are ahead of us as our knowledge of TICE and its therapeutic role in CVD keeps expanding.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest