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REVIEW

Intestinal cholesterol secretion: future clinical implications

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ABSTRACT

Together with the liver, the intestine serves as a homeostatic organ in cholesterol metabolism. Recent evidence has substantiated the pivotal role of the intestine in reverse cholesterol transport (RCT). RCT is a fundamental antiatherogenic pathway, mediating the removal of cholesterol from tissues in the body to the faeces. In humans, faecal cholesterol elimination via the RCT pathway is considered to be restricted to excretion via the hepatobiliary route. Recently, however, direct trans-intestinal excretion of plasma-derived cholesterol (TICE) was shown to contribute substantially to faecal neutral sterol (FNS) excretion in mice. TICE was found to be amenable to stimulation by various pharmacological and dietary interventions in mice, offering new options to target the intestine as an inducible, cholesterol-excretory organ. The relevance of TICE for cholesterol elimination in humans remains to be established. There is, however, emerging evidence for the presence of TICE in human (patho) physiology. This review discusses our current understanding of TICE and its novel therapeutic potential for individuals at increased risk of cardiovascular disease.

KEYWORDS

Reverse cholesterol transport, intestine, cardiovascular disease

INTRODUCTION

In the human body, cholesterol homeostasis is tightly regulated. This is not only of physiological importance, but also bears clinical relevance, since excessive cholesterol accumulation in the arterial wall invariably leads to the development of atherosclerotic cardiovascular disease (CVD). Although the inhibition of cholesterol synthesis by statins has resulted in a powerful reduction of CVD risk with a mean relative reduction of 25%,[1] there is still an unmet need for additional effective therapies to further reduce the residual CVD risk. In the past decade, research has mainly focussed on high-density lipoprotein cholesterol (HDL-C) raising therapies,[2,3] because of the strong inverse relationship between plasma HDL-C concentrations and CVD risk in epidemiological studies.[4-7] However, recent studies, aimed at increasing plasma HDL-C concentrations, have not substantiated a significant CVD risk reduction.[7-10] Hence, rather than aiming for an increase of HDL-C concentration, current research is focussed on elucidating and, if feasible, quantifying the mechanisms contributing to the atheroprotective properties of HDL-C.

The most established protective function of HDL-C is its role in the reverse cholesterol transport (RCT). This process was originally defined as the efflux of cholesterol from peripheral tissues, including arterial intra-plaque macrophages, subsequent transport in the plasma and uptake by the liver, followed by biliary secretion and elimination via the faeces.[11] Faecal excretion is the predominant way for eliminating cholesterol, because apart from conversion to bile acids, cholesterol cannot be catabolised to a significant extent within the human body. The classical RCT concept rests on two principles: 1. HDL-C is the primary lipoprotein involved in RCT and 2. biliary secretion is the sole route for intestinal elimination of plasma-derived cholesterol. In view of recent findings, both of these principles need to be reconsidered. The first is beyond the scope of this review. In short, in contrast to the current consensus, several studies have shown that plasma HDL-C levels do not determine biliary or faecal excretion of cholesterol in mice,[12-14] whereas

studies in humans have yielded conflicting results.[15-18] This review handles the second paradigm, the obligatory role of hepatobiliary cholesterol secretion in RCT. This historical concept has recently been challenged by studies in mice, indicating the existence of direct trans-intestinal cholesterol excretion (TICE) as an alternative cholesterol-eliminating pathway.

TRANS-INTESTINAL CHOLESTEROL EXCRETION: ANIMAL STUDIES

Cholesterol destined for hepatobiliary cholesterol secretion is taken up at the basolateral side of the hepatocyte via a number of lipoprotein receptors and is subsequently secreted at the canalicular membrane by a not fully elucidated secretion process, mediated for the largest part by the ATP-binding cassette G5/G8 (abcg5/g8) transporter.[19] If hepatobiliary cholesterol secretion were the primary route for cholesterol elimination, then inhibition of abcg5/g8 could be expected to result in extreme reductions of faecal neutral sterol (FNS) excretion. Interestingly, *abcg5* and *abcg8* double-knockout mice did not show these expected reductions in FNS loss.[20,21] Similar observations were made in other murine models of impaired hepatobiliary cholesterol secretion.[22-25] These studies unambiguously point towards the existence of an alternative, non-biliary cholesterol excretion pathway, at least in mice with genetically hampered hepatobiliary cholesterol secretion.

The concept of a non-biliary cholesterol excretion route is not novel. Already in 1927, it was demonstrated that FNS loss was paradoxically increased in dogs undergoing surgical bile diversion, as compared with control dogs.[26] These early findings were confirmed in a replication study in 1973,[27] as well as in studies in bile-diverted rats.[28,29] Similar observations have been made in the human situation, as described below.

More recently, additional murine intestinal perfusion studies and *in vivo* stable isotope studies substantiated that this alternative TICE route is also present in mice with intact hepatobiliary secretion and enterohepatic cycling.[30,31] In these studies, TICE accounted for roughly 20-33% of FNS loss. Moreover, the intestinal perfusion studies showed that plasma cholesterol can directly traverse the small intestine in a basolateral to apical direction, stimulated by the luminal presence of bile salt and phospholipid acceptors.[32] Furthermore, TICE was found to occur predominantly in the proximal part of the small intestine.[25,30] Importantly, a recent study showed that faecal excretion of macrophage-derived cholesterol can also proceed in the absence of biliary sterol secretion, suggesting that TICE can also mediate reverse cholesterol transport from cholesterol-loaded macrophages.[33] This implies that TICE may have antiatherogenic effects. However, a similar study could not confirm these results, for as yet unknown reasons.[34] Hence, it remains to be established whether induction of cholesterol elimination via TICE results in inhibition of atherosclerosis progression.

The molecular mechanisms underlying TICE are not fully understood. Hence, it is not known whether TICE is an active, transporter-mediated metabolic process. In order to effectively target this pathway, the following items need to be addressed: characterisation of plasma donor particles delivering cholesterol to the intestine for subsequent excretion via TICE; identification of transporters located at the basolateral membrane of intestinal cells, involved in the uptake of cholesterol destined for intestinal excretion; elucidation of intracellular trafficking mechanisms by which cholesterol is transported towards the apical membrane of enterocytes; identification of all apically located transporters and potential luminal acceptors which facilitate the excretion of cholesterol to the enteric lumen.

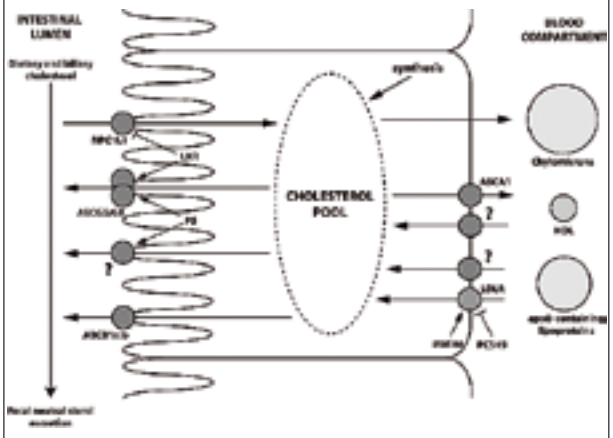
Thus far, some progress has been made, which has been the focus of recent comprehensive reviews.[35-37] The most important findings are summarised below (see also *figure 1*).

Despite its classical role in RCT, several studies indicate that plasma HDL is not the donor particle delivering plasma cholesterol for elimination via TICE. *Abca1* and *apoA1*-deficient mice, expressing negligible plasma HDL-C concentrations, exhibit normal or increased FNS excretion under normal conditions[12-14] and intestinal secretion of radiolabelled plasma-derived cholesterol was unaltered in *abca1*-/- mice as compared with their wild-type littermates.[38]

Instead, a recent review provides evidence to show that very-low-density lipoprotein (VLDL) remnants or further catabolic products of VLDL may serve as plasma donor particles delivering cholesterol for TICE.[35] In line, previous kinetic studies established a relatively high uptake rate of LDL cholesterol (LDL-C) into the intestine.[39] The LDL receptor (LDL-R) or one of the other receptors in the LDL-R family have been proposed as the basolateral transporters that mediate TICE.[35,40] However, experiments in *ldl-r*-/- mice failed to substantiate this concept.[25,35] Further studies are required to elucidate the intracellular itinerary of cholesterol destined for excretion via TICE.

The abcg5/g8 transporter, located at the brush border membrane of the small intestine, is likely to facilitate the last step of TICE. This is supported by several murine studies using various methodologies to quantify TICE.[31,41-43] In contrast, abcg5/g8 function was not found to affect TICE as measured in intestinal perfusion,[30] most likely reflecting methodological differences. Hence, TICE cannot be fully attributed to the activity of abcg5/g8, as a significant amount of TICE is still present in *abcg5* or *abcg8*-deficient

Figure 1. *Schematic representation of cholesterol fluxes in enterocytes related to the TICE pathway. The cholesterol pool of the intestinal cell is fuelled by uptake from the intestinal lumen via apically localised NPC1L1, endogenous synthesis, and via uptake of cholesterol from HDL and apoB-containing lipoproteins on the basolateral side (blood compartment). Apically, the main contributor to TICE is the ABCG5/G8 heterodimer; ABCB1a/b might also play a role as well as an additional route that has not yet been identified. On the basolateral side, the main cholesterol donors for TICE seem to be apoB-containing lipoproteins in a pathway that is likely to involve modulation of LDL-R expression. TICE can be increased by LXR activation as well as dietary plant sterols, partly dependent on functional ABCG5/G8 expression. The role of different intracellular pathways of cholesterol trafficking connecting basolateral uptake and apical secretion is currently unclear*



ABC = ATP-binding cassette; apoB = apolipoprotein B; HDL = high-density lipoprotein; LDL-R = low-density lipoprotein receptor; LXR = liver X receptor; NPC1L1 = Niemann-Pick C1 Like 1; PCSK9 = pro-protein convertase subtilisin kexin type 9; PS = plant sterols; TICE = transintestinal cholesterol excretion. (Adapted from: Tietge UW, Groen AK. Role the TICE?: Advancing the concept of transintestinal cholesterol excretion. Arterioscler Thromb Vasc Biol. 2013;33:1452-3).

mice. Other apically located proteins are likely to be involved. In fact, a recent report suggests that the abcb1a/b protein may serve as an apical excretory transporter in TICE.[40] Finally, it is plausible that acceptors in the intestinal lumen are required for cholesterol excretion via TICE. Bile acids and phospholipids in the intestinal lumen have been shown to stimulate the amount of cholesterol excreted via TICE.[30,32] The dependence on phospholipids is analogous to hepatic cholesterol secretion into the bile.[23] In the absence of these acceptors, only a small mass of TICE could be observed, which was probably attributable to shedding of enterocytes. Finally, the Niemann-Pick C2 (NPC2) protein was recently found to stimulate abcg5/g8-dependent biliary cholesterol secretion without affecting the abcg5/g8-independent pathway.[44,45] Although speculative, NPC2 might function as an acceptor for TICE mediated by abcg5/g8. Additional studies focusing on these acceptors might yield therapeutic interventions that would not require systemic distribution.

TRANS-INTESTINAL CHOLESTEROL EXCRETION: HUMAN STUDIES

The extent to which TICE contributes to faecal cholesterol elimination in humans remains to be established. Until recently, indications of the presence of TICE in human physiology were predominantly based on studies in patients with bile fistulae. In patients with complete biliary obstruction, a substantial portion of faecal sterols was found to be of non-dietary origin[46] and in another study in bile-diverted patients, the intestinal mucosa was found to secrete 250-400 mg of cholesterol per day.[47] A human intestinal perfusion study corroborated the presence of TICE, showing that approximately 44% of total FNS output originated from non-biliary origin.[48] These and a number of other reports[49-51] were mostly disregarded, likely pertaining to the small series of observations and the study limitations of the bile-diversion conditions. These include hampered cholesterol absorption and strongly upregulated cholesterol and bile acid synthesis. Furthermore, limitations of intestinal perfusion studies may have contributed, such as the absence of food, biliary and pancreatic components in the rinsed and perfused intestinal segments, together with the specific composition of the perfusate, which may have influenced the excretory capacity of enterocytes. Hence, studies on the non-biliary cholesterol excretion route were not pursued, until the more recent animal studies described above. Moreover, recent *in vitro* experiments using jejunal explants from humans showed activity of the TICE pathway for the first time in humans.[40] In these experiments, TICE depended on the presence of oxygen and was significantly decreased at low temperatures, which suggests that TICE is an active metabolic process.

Although the currently available human data collectively lend support to the presence of TICE in human cholesterol metabolism, a definite answer to this question has remained elusive. This is largely due to the technical challenges faced to reliably estimate this flux in humans *in vivo*, which requires simultaneous assessment of cholesterol absorption, biliary secretion and FNS excretion. We have recently attempted to quantify TICE in a population of mildly hypercholesterolaemic humans, by combining our previous experience from validated stable cholesterol isotope methodologies in mice[31] and humans.[15,52,53] Our unpublished data indicate that TICE is indeed present in human physiology and that it is sensitive to pharmacological stimulation, as described below.

TRANS-INTESTINAL CHOLESTEROL EXCRETION: FUTURE THERAPEUTIC POTENTIAL

The TICE pathway was found to be sensitive to various forms of dietary and pharmacological activation. However, at present, this is mostly confined to preclinical studies.

Liver-X-receptor agonists

Liver X nuclear receptors (LXRs) play a central role in cholesterol metabolism. Upon activation, LXRs induce expression of a series of genes that are involved in cholesterol efflux, absorption, transport and excretion.[54] Consistently, LXRs limit the development of atherosclerosis in mice and are therefore considered promising therapeutic targets for CVD risk reduction.[55] However, activation of LXRs concurrently promotes hepatic *de novo* lipogenesis, steatosis, and hypertriglyceridemia via direct activation of the sterol regulatory element-binding protein-1c (*SREBP-1c*) gene and fatty acid synthesis pathways.[56] LXR agonists were found to stimulate TICE up to sixfold in murine studies using different experimental methodologies.[23,30,31]

Recently, intestine-specific LXR agonists, which evade the unfavourable LXR-mediated effects on hepatic lipogenesis, have been developed. Studies indicate that intestine-specific activation of LXR, either genetic[57] or pharmacological,[58] is crucial for LXR-induced atheroprotection. Although it is tempting to suggest that TICE underlies parts of these favourable sequelae, a study which directly shows that TICE is stimulated by intestine-specific LXR agonists has not yet been reported. Finally, although promising in animal studies, the development of LXR-targeted drugs has largely been discontinued due to observations of marked increases in plasma apoB containing lipoproteins and/or a marked liver-steatotic response. To the best of our knowledge, there are no ongoing trials with intestine-specific LXR agonists. Hence, clinical studies evaluating their effects on TICE and atherosclerosis are not expected in the very near future.

Ezetimibe

Ezetimibe inhibits intestinal cholesterol absorption[59] in both mice and men, accomplished through inhibition of the Niemann-Pick C1 Like 1 (NPC1L1) transporter.[60] Despite a compensatory increase in endogenous cholesterol biosynthesis,[60] ezetimibe monotherapy lowers plasma LDL-C concentrations by approximately 15-20%.[59]

Ezetimibe has been shown to stimulate RCT from macrophages in mice, via as yet unidentified mechanisms.[61,62] Furthermore, when assessing cholesterol balance in ezetimibe-treated mice, the enhancement in FNS excretion cannot be attributed to cholesterol absorption inhibition or increased biliary cholesterol secretion alone.[41] In line, it has been suggested that ezetimibe might stimulate FNS excretion through stimulation of TICE,[63] although this was contradicted by another murine intestinal perfusion study.[64] Yet, our unpublished results of *in vivo* stable isotope studies in both mice and men showed a striking effect of ezetimibe on TICE [unpublished results, Jakulj, Stroes, Groen].

The underlying mechanisms by which ezetimibe might stimulate TICE are unknown. We speculated that the inhibition of NPC1L1 disturbs normal intracellular vesicle trafficking leading to increased transport of cholesterol to the apical membrane of the enterocytes.[41] Another possibility is that ezetimibe exerts its stimulatory effect on TICE by manipulation of the intraluminal bile acid and phospholipid content.[32,65,66]

Although our findings suggest an alternative mode by which ezetimibe might reduce plasma cholesterol concentrations and possibly reduce CVD risk, the latter issue is still precarious. Despite preclinical evidence that ezetimibe is atheroprotective,[67] to date, clinical studies have not been able to substantiate this: ezetimibe failed to regress carotid intima media thickness (cIMT) progression in patients with familial hypercholesterolaemia in the ENHANCE trial[68] and was found to be inferior to niacin in patients with coronary heart disease in the ARBITER-6 HALTS trial.[69] Next to major methodological disadvantages,[68,70] several off-target effects,[71] as well as upregulation of HMG-CoA reductase expression,[72] have been proposed as potential explanations. However, in the ARBITER-6 HALTS study, ezetimibe did hamper cIMT progression in statin-treated patients with fairly low LDL-C concentrations, who would thereby not likely to be considered for ezetimibe add-on therapy.[69] Furthermore, not all cIMT trials investigating ezetimibe have been negative.[73] A large clinical study of 18,000 patients, the IMPROVE-IT trial, is underway to determine whether additional cholesterol lowering by ezetimibe on top of statins can be translated into a reduction in cardiovascular event rate.[74] Although this trial started in 2005 and the results were expected in 2011, outcomes are still awaited, supposedly due to recruitment of additional patients after an unfavourable interim analysis. This trial is conducted in patients who have suffered from an acute coronary syndrome and who expressed low LDL-C concentrations at baseline, as inclusion of patients with higher LDL-C concentrations would not have achieved guideline-recommended LDL-C concentrations under the trial protocol, which would have been ethically unacceptable. Hence, it is conceivable that no additional benefit can be gained in this population, if ezetimibe's effect on atherosclerosis is causally related to plasma LDL-C reductions alone. Release of the study outcomes has been postponed until September 2014 (ClinicalTrials.gov:NCT00202878).

PCSK9

Proprotein convertase subtilisin kexin type 9 (PCSK9) is a secreted protein that reduces the amount of LDL-R at the cell surface of primarily the liver. PCSK9 circulates in the blood and binds the extracellular domain of the LDL-R to produce post-translational down-regulation of this receptor in lysosomes.[75] Loss-of-function mutations in the *PCSK9* gene result in 15-18% reductions in plasma LDL-C concentrations and carriers of these mutations express a 47-88% reduction in CVD risk.[76]

Next to the liver, PCSK9 is abundantly expressed in the intestine and it has been shown that PCSK9 modulates cholesterol transport and metabolism, as well as production of apoB-containing lipoproteins, in intestinal cells.[77] A recent report by the same research group revealed that PCSK9 is a repressor of TICE and that acute repression depended on a functional LDL-R.[40]

These findings may be of clinical importance, as Phase I and II PCSK9-inhibiting treatment modalities, such as single-stranded antisense DNA-like oligonucleotides or double-stranded small interference RNA, have shown promising results in terms of LDL-C lowering.[75] Phase III trials with longer duration and larger patient populations are currently underway, which should also establish whether PCSK9 inhibition reduces cardiovascular event rate in humans.

Plant sterols

Plant sterols are not endogenously synthesised by humans, but are strictly derived from the diet. They perform functions in plant cells similar to those of cholesterol in mammalian cells. Campesterol and sitosterol are the most abundant ones. They share a high degree of structural similarity with cholesterol, but are much more hydrophobic. Plant sterols are present in small amounts of fruits, vegetables, nuts, seeds and edible oils; marketed sources are primarily derived from soybean and pine tree oil. Total dietary plant sterol consumption in the average Western diet is 150-350 mg per day.[78,79] Daily consumption of 2g of plant sterols is associated with LDL-C reductions varying from 4-15% in hypercholesterolaemic or normocholesterolaemic adults.[80]

Plant sterols are thought to displace cholesterol from incorporation into micelles, thereby limiting cholesterol absorption in the intestinal lumen by approximately 25-36%.[81] However, additional cholesterol-lowering mechanisms have been postulated.[82] Interestingly, the cholesterol-lowering effects of plant sterol consumption were recently ascribed to stimulation of intestinal cholesterol excretion via TICE, as plant sterol feeding resulted in a sixfold induction of TICE in wild-type mice.[43] This is supported by a recent crossover plant sterol feeding trial in 18 adults, who in random order consumed dietary plant sterols from negligible (0 mg) to high (2g) amounts, resulting in a dose-dependent increase in FNS output, which could not be explained by the corresponding reductions in measured cholesterol absorption.[83]

The mechanisms by which plant sterols could stimulate TICE are currently unknown. Several mechanisms have been suggested, including both LXR-dependent and -independent mechanisms.[82] It is less likely that the stimulation of TICE is LXR-mediated, as plant sterols did not alter LXR target genes in the study by Brufau *et al.*[43] and studies investigating plant sterols as possible ligands of LXR have been conflicting.[21,84,85] A possible LXR-independent mechanism might include interference of plant sterols with cholesterol trafficking within the enterocyte, as plant sterols have been shown to affect expression of genes encoding proteins of the annexin family, which are involved in the regulation of membrane properties.[86] Besides studies aiming to unravel the underlying molecular mechanisms, human studies to assess the effect of plant sterols on TICE are also lacking at present.

CONCLUSIONS

In conclusion, trans-intestinal cholesterol excretion might serve as an attractive future target for LDL-C lowering and CVD reduction, provided underlying molecular mechanisms are elucidated. Although promising, the therapeutic potential of targeting the TICE pathway is to date confined to preclinical studies and it is unknown whether pharmacological targeting of the TICE pathway will also yield a clinical benefit. Available interventions that have been shown to stimulate TICE and may therefore warrant further clinical evaluation include ezetimibe, PCSK9-inhibitors and plant sterols.

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