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# A New Model of Reverse Cholesterol Transport: EnTICEing Strategies to Stimulate Intestinal Cholesterol Excretion

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## Abstract

Cardiovascular disease (CVD) remains the largest cause of mortality in most developed countries. Although recent failed clinical trials and Mendelian randomization studies have called into question the high density lipoprotein (HDL) hypothesis, it remains well accepted that stimulating the process of reverse cholesterol transport (RCT) can prevent or even regress atherosclerosis. The prevailing model for RCT is that cholesterol from the artery wall must be delivered to the liver where it is secreted into bile before leaving the body through fecal excretion. However, many studies have demonstrated that RCT can proceed through a non-biliary pathway known as transintestinal cholesterol excretion (TICE). The goal of this review is to discuss the current state of knowledge of the TICE pathway, with emphasis on points of therapeutic intervention.

### Keywords

cholesterol; lipoprotein; bile; reverse cholesterol transport; transintestinal cholesterol excretion

## THE EVOLVING LANDSCAPE OF RCT

Atherosclerosis and associated cardiovascular disease (CVD) remains the largest cause of mortality in developed countries [1]. Despite widespread use of statin drugs to reduce levels of low density lipoprotein (LDL), CVD-associated mortality and morbidity has been reduced by only ~30% [1], demonstrating a clear need for better therapeutic strategies. Elevation of high density lipoprotein (HDL) function is thought to be an attractive

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therapeutic strategy [2]. However, recent clinical trials [3,4] and Mendelian randomization studies [5] have failed to show clinical benefits of HDL cholesterol elevation, calling into question the importance of HDL cholesterol as a surrogate marker of protection from atherosclerosis [2]. Both proponents and critics alike of the “HDL hypothesis” agree on one thing – further studies are needed to understand the mechanism regulating the fundamental process of HDL-driven reverse cholesterol transport (RCT). The prevailing model for RCT is that cholesterol from the artery wall is delivered to the liver via HDL, where it is then secreted into bile before leaving the body through fecal excretion [6–8]. However, we and others have recently demonstrated that RCT can also proceed through a non-biliary pathway known as transintestinal cholesterol excretion (TICE), which involves the direct secretion of plasma lipoprotein-derived cholesterol by the small intestine [9–18]. Although mechanisms regulating the classic biliary pathway of RCT have been well defined [19–23], almost no mechanistic information exists for the non-biliary TICE pathway [10]. Therefore, the purpose of this review is to discuss the most up to date understanding of the non-biliary TICE pathway, with particular emphasis on strategies to stimulate intestinal cholesterol excretion for the prevention or treatment of atherosclerotic CVD.

RCT is a multi-organ pathway that facilitates the removal of excess cholesterol from the body, and this homeostatic pathway is conserved across a wide range of organisms [6–8,24–26]. Importantly, elevated flux through the RCT pathway is thought to protect against CVD primarily by facilitating cholesterol removal from macrophage foam cells within the atherosclerotic plaque [6–8]. The vast majority of literature assumes that RCT involves the sequential movement of macrophage-derived cholesterol from peripheral tissues to the liver for excretion into bile and subsequent loss through the feces [6–8]. In this well accepted model, biliary secretion of cholesterol is requisite for the final step of RCT, namely fecal cholesterol and bile acid excretion [6–8]. Relevant to this review, emerging evidence supports an unexpected role for the small intestine in actively secreting plasma-derived cholesterol through a process that does not rely on hepatobiliary cholesterol secretion [9–18]. The identification of this non-biliary pathway largely stems from multiple observations where biliary cholesterol secretion does not predict the amount of cholesterol in the feces [10,27–32]. Given that several recent reviews have been written surrounding the mounting evidence for the non-biliary TICE pathway [10,27–32], we refer the reader to these excellent resources for a historical perspective. Briefly, previous studies in humans [18,33–38], dogs [39,40], rats [41–43], and mice [9–18] have unequivocally demonstrated the existence of a non-biliary pathway for RCT, especially under conditions where biliary cholesterol loss is surgically or genetically interrupted. However, there is still controversy in the field regarding the relative importance of the classic biliary and non-biliary pathways for RCT. Furthermore, there is incomplete understanding regarding the therapeutic utility of targeting one or the other pathway. Here we wish to highlight the consensus and controversies surrounding both biliary and non-biliary RCT pathways, and critically discuss the realistic potential for targeting the non-biliary TICE pathway for prevention or treatment of CVD.

Although most in the field agree that a non-biliary RCT pathway exists, there has been some disagreement and confusion as to the relative contributions of biliary and non-biliary pathways. When considering the relative contribution of biliary versus non-biliary routes to RCT flux, it is important to discuss this in the context of normal physiology versus

pathophysiologic conditions where biliary cholesterol movement to the intestine is interrupted. First, under normal physiological conditions, the biliary route predominates while the non-biliary pathway typically makes up less than ~30% of the total cholesterol found in the feces [10,27–32]. For instance, in chow-fed mice the non-biliary TICE pathway accounts for approximately 20–33% of total fecal neutral sterol loss [11,14]. Although validation studies are needed, early intestinal perfusion studies estimated as much as 44% of fecal cholesterol loss originated from non-biliary origins in humans [34]. Although these estimations confirm the assumption that the biliary route is the predominant RCT pathway under normal physiological conditions, the non-biliary TICE pathway is highly dynamic and can be stimulated by both pathophysiologic and pharmacologic stimuli. For example, in genetically modified mouse models that lack the ability to normally secreted cholesterol into bile (ABCG5/G8$^{-/-}$, Mdr2$^{-/-}$, and NPC1L1$^{-\text{LiverTg}}$), or under conditions where the common bile duct is surgically excluded from the small intestine (acute or chronic biliary diversion), fecal cholesterol loss remains either unchanged or in some cases increased [9,19,22,33,34,39,40,44,45]. These studies strongly suggest that under the pathophysiologic condition of biliary cholesterol insufficiency, the non-biliary TICE pathway can fully compensate to maintain normal levels of fecal cholesterol loss. Another important implication from these studies is that when biliary cholesterol movement to the intestine is blocked, there must be a signaling mechanism to instruct the liver to stimulate TICE to maintain cholesterol homeostasis. In addition to dynamic regulation under pathophysiologic conditions, the non-biliary TICE pathway can also be stimulated pharmacologically [10,27–32], which will be discussed under points of therapeutic intervention later in this review. Collectively, fecal cholesterol disposal (i.e. the end result of RCT) relies on a dynamic interplay between both biliary and non-biliary pathways, and the contribution of each can be quite different under physiological, pathophysiological, and pharmacological conditions. As cholesterol lowering drug discovery efforts move forward, it will be extremely important to consider the shared and distinct mechanisms regulating both biliary and non-biliary branches of the RCT pathway.

## THE NEW INTEGRATED MODEL OF RCT

Although the non-biliary TICE pathway is highlighted here, it is not the only concept challenging the widely accepted hepatobiliary model for RCT [6–8]. In fact, several lessons learned during the past decade have suggested that substantial revisions are needed to the original model of RCT proposed by John Glomset and colleagues [46,47]. Unfortunately, many of these paradigm-shifting discoveries are largely ignored, perpetuating a theoretical RCT model that is more based on dogma than experimental evidence. For instance, it has long been assumed that HDL are rate limiting for RCT flux of cholesterol out of the body, because it is predicted that they deliver peripheral cholesterol to the liver for biliary secretion and ultimately fecal excretion [7,8]. However, recent discoveries highlight that this assumption is only partially correct. Without dispute, lipid poor apolipoprotein AI (apo-AI) or spherical HDL can initiate RCT by promoting the efflux of cholesterol from many cells including macrophage foam cells in the artery wall [8,48,49]. Likewise, there is strong evidence that HDL cholesterol can be efficiently delivered to the liver, primarily via the action of scavenger receptor class B type I (SR-BI) [50–52]. However, there is now

evidence from several well-respected laboratories that HDL-mediated cholesterol efflux and HDL-driven centripetal movement of cholesterol back to the liver does not correlate with how much cholesterol is lost in bile or the feces [53–56]. In fact, mice genetically lacking apoA-I or ATP-binding cassette transporter AI (ABCA1) (i.e. mice that have extremely low circulating levels of HDL) have normal biliary and fecal cholesterol loss [53–56]. These data provocatively suggest that circulating HDL cholesterol levels have little to do with the amount of cholesterol actually leaving the body through the bile or feces, prompting revision of the classic RCT model. Although typically excluded in most RCT models, it is likely that apoB-containing lipoproteins play quite a substantial role in RCT [58–61]. It is important to note that the activity of cholesteryl ester transfer protein (CETP) plays a major role in RCT, given its ability to transfer HDL-derived cholesteryl esters into triglyceride-rich apolipoprotein B (apoB)-containing lipoproteins [58–61]. Therefore, the relative contributions of HDL and apoB-containing lipoproteins to biliary and fecal cholesterol loss may be markedly different in CETP-containing species like humans [58–61]. Collectively, progress made within the last decade clearly demonstrates that the dogmatic model of RCT requires revision.

With such progress in mind, here we propose a new integrated model for RCT considering the shared and distinct branch points for biliary and non-biliary RCT (Figure 1). Much like the original model proposed by Glomset and Norum [46,47], this updated model begins with the *de novo* synthesis of HDL. There is now overwhelming evidence that the process of RCT is initiated by the ABCA1-dependent lipidation of apoA-I to form nascent pre-beta HDL particles primarily in hepatocytes and enterocytes [62,63]. These liver and intestine-derived pre-beta HDL particles can then accept free cholesterol from peripheral tissues, and be further matured by the enzymatic actions of lecithin:cholesterol acyltransferase (LCAT), phospholipid transfer protein (PLTP), and other enzymes into spherical HDL particles [64–66]. This stepwise HDL maturation process collectively facilitates the centripetal flux of cholesterol to the liver, while avoiding apoA-I/HDL catabolism by the kidney [62–69]. Once matured with both free and esterified cholesterol cargo, HDL is cleared by the liver either via SR-BI-dependent selective uptake [51,52] or by holoparticle uptake mechanisms involving proteins such as F1F0-ATPase [70] and the nucleotide purinergic receptor P2Y G protein receptor 13 (P2Y13) [71–73]. Importantly, in CETP-containing species, a large portion of HDL's cholesteryl ester cargo can be transferred to apoB-containing lipoproteins by CETP, which are taken up by the liver by hepatic low density lipoprotein receptors (LDLr) contributing to overall RCT flux [60,61]. All steps up to this point represent well understood pathways in RCT, and this shared centripetal flux collectively delivers cholesterol to the liver where this cargo can then branch into either biliary or less well understood non-biliary pathways. As classically understood, once delivered to the liver, a large portion of the RCT-derived cholesterol pool can be secreted into bile via the actions of proteins such as ATP-binding cassette transporters G5 and G8 [19–20] and ATP8B1 [23]. An additional key determinate of biliary cholesterol flux is hepatic SR-BI, given that SR-BI can facilitate directional (basolateral to apical) trafficking of cholesterol in polarized cells [74–77] and can facilitate biliary cholesterol secretion *in vivo* [78]. Once cholesterol is secreted into bile a large portion of this pool is physically delivered to the lumen of the small

intestine via the common bile duct, where it can ultimately provide substrate for fecal cholesterol loss.

Alternatively, the liver can initiate the non-biliary TICE pathway to eliminate excess cholesterol, especially under conditions where biliary cholesterol secretion is limited [9–18]. Current evidence suggests that the non-biliary branch of RCT can be initiated by either re-uptake of biliary cholesterol via the cannalicular sterol transporter Niemann-Pick C1-like 1 (NPC1L1) [9,22], or by blocking cholesterol acyl-CoA:cholesterol acyltransferase 2 (ACAT2)-driven cholesterol esterification [13,79]. Both of these conditions are expected to cause an accumulation of hepatic free cholesterol, yet under these conditions the excess free cholesterol is repackaged onto nascent apoB-containing lipoproteins, which are ultimately secreted from the liver into the bloodstream [9,13,22,79]. These liver-derived apoB-containing lipoproteins are then recognized by the proximal small intestine through lipoprotein receptors such as LDLr [18], and likely other mechanisms given that TICE can still occur in $LDLr^{-/-}$ mice [13]. Importantly, there is no evidence that the HDL receptor SR-BI is involved in non-biliary TICE [80]. Once cleared by the proximal small intestine, the trafficking itinerary of TICE-derived cholesterol within the intestinal enterocyte is not well understood. However, given that apoB-containing lipoproteins are the TICE donor particles, it is tempting to speculate that the trafficking itinerary would involve endosomal/lysomal compartments. Once the free cholesterol is liberated from TICE lipoproteins in the intestine, this cholesterol can be effluxed across the apical membrane via the actions of ATP binding cassette trasporters ABCG5/ABCG8 [17,45,81] and ABCB1a/b [18]. Collectively this TICE flux through the intestine, coupled with biliary cholesterol secretion, and dietary cholesterol make up the sum total of cholesterol available for excretion into the feces (Figure 2). As will be discussed later, once within the lumen of the intestine, RCT-derived cholesterol can be efficiently re-absorbed via the actions of intestinal NPC1L1 [82]. The process of intestinal NPC1L1-driven cholesterol absorption directly opposes biliary and non-biliary RCT flux [83] (Figure 2). Within this new integrated model of RCT (Figure 1), there are several points of therapeutic intervention that warrant discussion.

## Points of therapeutic intervention

**Common HDL-driven centripetal flux of cholesterol back to the liver—**The common component of RCT encompasses the multistep process of HDL-driven centripetal flux of cholesterol from peripheral tissues back to the liver (Figure 1) [7,8]. This initial portion of centripetal flux is what most people define as RCT [7–9], but HDL-driven movement of cholesterol back to the liver is only one part of the RCT pathway. It is important to note that once HDL-derived cholesterol is delivered to the liver, a poorly understood sorting process occurs to dictate whether this new cholesterol pool is used locally or shunted into biliary or non-biliary RCT pathways for ultimate excretion into the feces to complete removal from the body (Figure 1). In the context of atherosclerosis, the common centripetal RCT pathway starts by removal of cholesterol from the macrophage foam cell in the artery wall, and ends by HDL-driven delivery of macrophage-derived cholesterol to the liver (Figure 1). Unequivocally, elevation of apoA-I or HDL can prevent or even regress established atherosclerosis in animal models and humans, and it is assumed that removal of excess cholesterol from lesional macrophages is a primary mechanism

supporting this atheroprotection [84–95]. However, apoA-I and HDL have many biological properties including their ability to suppress inflammation [96], regulate hematopoetic stem cell proliferation [97], and serve as circulating carriers of diverse cargo such as bioactive lipids, proteins/enzymes, hormones, vitamins and small non-coding RNAs [98–100]. Any of these diverse functions of HDL could potentially regulate the pathogenesis of atherosclerosis. Although apoA-I and HDL can stimulate cholesterol efflux from macrophage foam cells in culture, recent evidence suggests that genetic modulation of the early steps in HDL biogenesis has very minor or non-existent effects on RCT flux into the bile or feces [53–57,100,101]. First, mice with extremely low levels of HDL due to defects in synthesis or maturation (ABCA1$^{-/-}$, apoA-I$^{-/-}$, LCAT$^{-/-}$) have normal levels of RCT into bile and feces [53–57]. In a recent elegant study, John Parks and colleagues demonstrated that genetic deletion of ABCA1 in hepatocytes (the primary site of HDL biogenesis) had no effect on macrophage RCT into bile or feces, and also did not alter atherosclerosis progression in LDLr$^{-/-}$ mice [100]. Likewise, maturation of HDL driven by LCAT-mediated cholesterol esterification is not a major determinant of macrophage RCT into bile or feces in mice [101]. Collectively, these recent insights show that although centripetal flux of cholesterol to the liver is generally considered to determine the quantity of cholesterol removed from the body through bile and feces [7,9], it is the subsequent steps within the liver that dictate the amount of cholesterol ultimately secreted into biliary and non-biliary excretory routes (i.e. the end result of RCT). Thus, future drug discovery efforts may benefit from targeting hepatic pathways that traffic cholesterol into either the biliary or non-biliary branch points of RCT (Figure 1).

**Increasing flux through the biliary pathway in the liver—**In the classic view of RCT [7–9], it is assumed that centripetal movement of cholesterol to the liver will result in the secretion of this HDL-derived cargo into bile, which will ultimately lead to fecal excretion. However, the assumption that HDL-derived cholesterol moves exclusively into bile is not strongly supported by recent findings in mice with altered HDL biogenesis [53–57,100,101]. As mentioned previously, mice lacking the ability to synthesize apoA-I-containing HDL have normal biliary and fecal cholesterol levels [53–57,100,101]. Instead of being primarily governed by HDL-driven RCT flux, biliary cholesterol secretion is under dynamic regulation by both protein-mediated and biophysical mechanisms [19–23,102,103]. The first stage of control for biliary cholesterol secretion is at the level of the HDL receptor SR-BI. SR-BI has the unique ability to facilitate directional (basolateral to apical) trafficking of cholesterol in polarized cells [74–77], and SR-BI-dependent selective uptake promotes biliary cholesterol secretion *in vivo* [78]. This ability of SR-BI to directionally traffic cholesterol across the hepatocyte likely plays a major role in linking centripetal RCT flux to biliary cholesterol secretion. Once cholesterol arrives at the cannalicular membrane (the site of bile formation), the major mechanism of biliary secretion involves protein-mediated translocation of sterols across the cannalicular membrane by the heterodimeric half-transporters ABCG5 and ABCG8 [19,20]. However, a small amount of cholesterol can still be secreted into bile in ABCG5/ABCG8$^{-/-}$ mice through alternative mechanisms involving ATB8B1 and diffusion [21,23]. Importantly, protein-mediated and diffusion-driven movement of cholesterol into bile requires the presence of a biliary acceptor “micelle” which is assembled during the simultaneous transport of bile acids and phospholipids into

bile by dedicated protein transporters such as bile salt export pump (BSEP) and multi-drug resistance protein 2 (Mdr2) [11,23,44,102]. Given the requirement of the “micellar” acceptor, the rate of biliary cholesterol secretion is highly dependent on the rate of bile acid and phospholipid secretion [11,23,44,102,103]. Once cholesterol is secreted into bile, another control mechanism exists in humans to prevent excessive cholesterol loss through the bile [22]. Much like its role in the small intestine, the sterol transporter NPC1L1 can transport newly secreted biliary cholesterol back into hepatocytes [22], and this re-uptake mechanism can instead initiate flux through the non-biliary TICE pathway (Figure 1). There have been a number of molecular targets identified controlling the flux of cholesterol into the bile, yet therapeutic interventions selectively targeting enhanced biliary secretion lack efficacy in atheroprotection and can cause unwanted side effects.

Like other concepts in the RCT field, the assumption that specifically increasing biliary cholesterol loss will protect against atherosclerosis is not strongly supported by experimental evidence. For instance, hepatocyte-specific overexpression of ABCG5 and ABCG8 results in ~2-fold increase in biliary cholesterol secretion, but this chronic increase in biliary cholesterol levels does not equate to protection against atherosclerosis in either apoE or LDLr knockout mice [104]. Subsequent studies have demonstrated that the elevated biliary cholesterol in these ABCG5/ABCG8 transgenic mice can be efficiently recovered by NPC1L1 in the small intestine, and this intestinal re-absorption pathway opposes the atheroprotection expected by removing cholesterol from the body [105]. These studies suggest that in order for drugs targeting enhanced biliary cholesterol secretion to efficaciously protect against atherosclerosis, they need to be given in combination with an intestinal cholesterol absorption inhibitor such as ezetimibe [104,105]. Another extremely important consideration when designing drugs to stimulate biliary cholesterol flux is potential deleterious side effects, given the critical role that biliary cholesterol secretion plays in gallstone formation [102,103]. It has long been known excessive biliary cholesterol secretion can result in supersaturation of bile, promoting cholesterol nucleation and gall stone formation [102,103]. Although most assume that RCT flux into bile is a fundamental pathway mediating atheroprotection [7–9], it is not guaranteed that targeted stimulation of biliary cholesterol loss will indeed result in a negative cholesterol balance due to intestinal absorption [104,105]. Further, there may be unwanted side effects of stimulating biliary cholesterol secretion such as cholesterol gallstones [102,103]. With these caveats in mind, all current data suggests that targeting the non-biliary branch of RCT holds untapped therapeutic potential with no predicted side effects. Although not discussed here due to space limitations, there is a large capacity of hepatic cholesterol to be converted into bile acids, which can ultimately contribute substantially to biliary RCT [6–8].

**Increasing flux through the non-biliary TICE pathway—**Although much is known regarding mechanisms regulating common centripetal RCT flux back to the liver and hepatobiliary secretion, we are still in our infancy in understanding the non-biliary TICE pathway. However, based on research performed within the last decade, there are several steps in the non-biliary TICE pathway that could be potentially therapeutically exploited.

**Hepatic lipoprotein production—**Through the function of ABCA1, the liver produces nascent HDL that upon maturation carries the majority of circulating HDL cholesterol [62]. Until recently it was widely believed that hepatic HDL production was an important contributor to RCT. However, Bi and colleagues found that $Ldlr^{-/-}$ mice with hepatocyte-specific deletion of ABCA1 had an ~50% reduction in HDL cholesterol but no change in either macrophage-to-feces RCT or aortic atherosclerosis development [100]. Moreover, it had been previously reported that mice with whole-body ABCA1 deficiency ($Abca1^{-/-}$), which have virtually no circulating HDL cholesterol, have normal biliary and fecal cholesterol levels [55,56]. Most recently, Vrins and colleagues also reported that the rate of TICE in $Abca1^{-/-}$ mice was not significantly different than that of wild-type littermates [106]. Thus, current data suggests that therapeutic strategies that raise hepatic HDL production will not likely stimulate TICE.

Unlike HDL, liver-derived apoB-containing lipoproteins appear to promote non-biliary RCT (Figure 1). We have previously reported that mice with hepatic knockdown of acyl-CoA:cholesterol acyltransferase (ACAT2), also known as sterol o-acyltransferase ($Soat2^{HKD}$) have normal biliary cholesterol secretion, but increased fecal neutral sterol excretion, suggesting increased TICE [13]. In this study, we found that the small intestine accumulated a greater amount of cholesterol originating from nascent VLDL from $Soat2^{HKD}$ compared to control mice [13]. In a follow up study, we have shown that acute $Soat2^{HKD}$ in cholesterol-fed mice promotes rapid increases in fecal neutral sterol loss without changes in biliary cholesterol levels, and this increased TICE flux was accompanied by acute elevation of circulating apoB-containing lipoproteins [79]. In addition, we recently tested the hypothesis that reduced hepatic VLDL secretion would limit TICE flux. To address this, $NPC1L1^{LiverTg}$ (a mouse model of chronically activated TICE) and littermate controls were treated with antisense oligonucleotides (ASOs) to knockdown the hepatic expression of microsomal triglyceride transfer protein (MTP), which is needed for proper lipidation and secretion of apoB-containing lipoproteins. Hepatic knockdown of MTP ($MTP^{HKD}$) significantly reduced hepatic triglyceride secretion but did not impact biliary cholesterol concentration [107]. Fecal neutral sterol excretion was significantly reduced with $MTP^{HKD}$ in $NPC1L1^{LiverTg}$ but not littermate controls leading to the conclusion that hepatic VLDL secretion is necessary for TICE [107]. Our conclusion was supported by the findings of Dikkers and colleagues who showed that macrophage-to-feces RCT was decreased in mice with hepatic MTP deficiency [108]. Thus, stimulating the secretion of hepatic apoB-containing lipoproteins may promote TICE. However, therapeutic targeting of this step in the non-biliary RCT pathway would likely be problematic since it may elevate circulating LDL levels and consequently increase the risk of atherosclerotic CVD.

**Intestinal lipoprotein uptake—**The uptake of cholesterol from high density lipoproteins (HDL) by the liver is an important step in the hepatobiliary RCT pathway. Hepatic overexpression of SR-BI, which mediates the selective uptake of HDL cholesterol, increases macrophage-to-feces RCT in mice [109]. Since SR-BI is also expressed in enterocytes, we determined whether increasing intestinal SR-BI would promote TICE [80]. $SR\text{-}BI^{hApoCIII\text{-}ApoAIV\text{-}Tg}$ mice that overexpress SR-BI in the small intestine, and to a lesser extent in the liver, have reduced HDL cholesterol but no change in cholesterol absorption

and fecal neutral sterol excretion [80]. SR-BI$^{hApoCIII\text{-}ApoAIV\text{-}Tg}$ mice were also treated with ezetimibe to block cholesterol absorption and potentially unmask an increase in TICE. However, SR-BI$^{hApoCIII\text{-}ApoAIV\text{-}Tg}$ mice and wild-type littermate controls had similar fecal neutral sterol excretion when treated with ezetimibe [80]. We also crossed NPC1L1$^{LiverTg}$ mice, which have increased TICE, with SR-BI$^{hApoCIII\text{-}ApoAIV\text{-}Tg}$ mice. The NPC1L1$^{LiverTg}$ and double transgenic mice had similar biliary cholesterol concentration, cholesterol absorption, and fecal neutral sterol excretion [80]. From these results, we concluded that intestinal SR-BI overexpression does not increase TICE. Collectively, we do not believe that TICE will be stimulated by therapies that increase SR-BI in the gut.

Since the current data indicates that liver-derived apoB-containing lipoproteins are responsible for supplying cholesterol for TICE, increasing intestinal LDL receptor (LDLr) expression should drive non-biliary RCT (Figure 1). In a recent study, Le May and colleagues showed that conditions stimulating LDLr expression, such as Pcsk9 deficiency and statin treatment, increased TICE in mice undergoing intestinal perfusion [18]. Unexpectedly, they also found that TICE was increased in Ldlr$^{-/-}$ mice [18]. We have also reported that Ldlr$^{-/-}$ with hepatic Soat2 knockdown have increased TICE flux [13]. Moreover, LXR activation has been shown to significantly increase TICE in mice [10,11,14], but at the same time nearly eliminates intestinal LDLr protein due to the action of the E3 ubiquitin ligase Idol [110]. These results indicate that stimulating LDLr expression should promote TICE, but also that other receptor systems feed cholesterol from apoB-containing lipoproteins into the non-biliary RCT pathway. Other members in the LDL receptor family such as LRP, VLDL receptor (VLDLR), and apoER2 are expressed in the small intestine [111,112], and could conceivably act as primary or secondary receptors for apoB-containing lipoproteins. It is also conceivable that like in the liver [113], heparin sulfate proteoglycans mediate the uptake of VLDL remnants and consequently support TICE. Currently, there are many unanswered questions concerning the intestinal receptors involved in TICE flux. Hence, defining the lipoprotein receptor systems that provide TICE-derived cholesterol to the small intestine has the potential to uncover new therapeutic targets.

**Intestinal specific LXR activation—**Systemic activation of liver X receptor (LXRs) with synthetic agonists has been reported to increase RCT by stimulating both the hepatobiliary and TICE pathways [9,11,14,113]. However, activation of LXRs in the liver increases *de novo* lipogenesis resulting in the unwanted side effect of hepatic steatosis [114]. Moreover, LXR agonists increase Idol in primate but not rodent liver consequently decreasing LDLr protein and elevating LDL concentration [115]. Because of these adverse effects, systematic LXR agonists have only been used to increase RCT in preclinical trials. Intestinal specific activation of LXRs may be one way to increase RCT and eliminate the side effects associated with systemic LXR agonists. Yasuda and colleagues reported that an intestinal specific LXR agonist was able to significantly increase macrophage-to-feces RCT [116]. In addition, Lo Sasso and colleagues [117] created a mouse model with constitutively active LXRα in enterocytes, and found that these animals had decreased cholesterol absorption and increased fecal excretion of macrophage-derived neutral sterol. In both studies, the heterodimeric sterol transporters ABCG5 and ABCG8 were increased

exclusively in intestine therefore leading to the conclusion that the elevation in macrophage RCT was the result of reduced cholesterol absorption [116,117]. However, it is also possible that increased ABCG5 and ABCG8 in enterocytes promote TICE. The recent study by Wang and colleagues highlights the possible role that ABCG5 and ABCG8 play in non-biliary RCT [45]. In this study, mice with liver (L-G5G8$^{-/-}$), intestinal (I-G5G8$^{-/-}$) or whole body (G5G8$^{-/-}$) knockout of ABCG5 and ABCG8 were created. In order to gauge the importance of hepatic and intestinal ABCG5/ABCG8 in RCT, the mice were injected with $^{3}$H-cholesterol, and the levels of $^{3}$H-cholesterol in bile and feces were measured. The percentage of $^{3}$H-cholesterol in the bile of the mice was qualitatively similar to that observed for biliary cholesterol mass (WT = I-G5G8$^{-/-}$ > L-G5G8$^{-/-}$ > G5G8$^{-/-}$). Reduced biliary cholesterol secretion undoubtedly contributed to the decrease in fecal $^{3}$H-cholesterol excretion for the L-G5G8$^{-/-}$ and G5G8$^{-/-}$ versus WT mice. I-G5G8$^{-/-}$ compared to WT mice also had decreased fecal $^{3}$H-cholesterol excretion, which could have resulted from increased cholesterol absorption. However, fractional cholesterol absorption was similar for I-G5G8$^{-/-}$ and WT mice. Therefore, the reduction in fecal $^{3}$H-cholesterol excretion for the I-G5G8$^{-/-}$ mice could have been due to decreased TICE. Collectively, these studies suggest that intestinal specific LXR agonists could promote RCT by decreasing cholesterol absorption and increasing TICE.

**Inhibition of cholesterol absorption with ezetimibe—**Ezetimibe is a clinically-approved drug used to treat hypercholesterolemia. It is well established that ezetimibe lowers LDL cholesterol by inhibiting NPC1L1 in the small intestine consequently reducing cholesterol absorption [82]. In species such as humans that express NPC1L1 in liver, ezetimibe may also decrease LDL cholesterol by blocking the recycling of biliary cholesterol by hepatocytes [22]. In theory, cholesterol coming from the hepatobiliary or transintestinal pathway could be absorbed by the small intestine prior to being excreted into the feces. Therefore, by blocking cholesterol absorption, ezetimibe should stimulate both biliary and non-biliary RCT. Several groups have shown in rodent models in which the biliary pathway should predominate that ezetimibe treatment significantly increases macrophage-to-feces RCT [83,118–120]. Moreover, ezetimibe appears to increase RCT in humans since ezetimibe stimulated the excretion of stable isotope labeled cholesterol that had been IV infused into hyperlipidemic patients [121]. There are two recent studies that have addressed whether ezetimibe increases RCT through the TICE pathway. To eliminate any contribution to RCT from the hepatobiliary pathway, Uto-Kondo et al performed bile duct ligations on ezetimibe-treated hamster and then measured macrophage-to-feces RCT [120]. Ezetimibe significantly increased fecal excretion of macrophage-derived $^{3}$H-neutral sterol in bile duct ligated hamsters. However, macrophage-to-feces RCT was stimulated to a much greater extent in sham operated compared to bile duct ligated animals. Therefore, it can be concluded that that ezetimibe can stimulate TICE but requires the hepatobiliary pathway to maximally stimulate RCT.

We previously reported that despite very low biliary cholesterol secretion, NPC1L1$^{\text{LiverTg}}$ mice have normal macrophage-to-feces RCT and therefore concluded that NPC1L1$^{\text{LiverTg}}$ mice have increased TICE [9]. We also found that ezetimibe treatment could normalize biliary cholesterol concentration in NPC1L1$^{\text{LiverTg}}$ mice [22]. To determine whether

inhibiting hepatic NPC1L1 with ezetimibe would facilitate macrophage RCT, Xie and colleagues created NPC1L1$^{LiverTg}$ mice lacking intestinal Npc1l1 (NPC1L1$^{LiverOnly}$) and fed the mice a low cholesterol diet, which caused the vast majority of excreted cholesterol to come from the either the hepatobiliary or TICE pathway [122]. Compared to Npc1l1 whole body knockouts (Npc1l1$^{-/-}$), the NPC1L1$^{LiverOnly}$ mice had extremely reduced biliary cholesterol as expected [122]. Unlike what we reported for NPC1L1$^{LiverTg}$ mice with intact intestinal NPC1L1 function, mass and macrophage-derived fecal neutral sterol excretion was significantly reduced in the NPC1L1$^{LiverOnly}$ compared to Npc1l1$^{-/-}$ mice [122]. Treatment of the NPC1L1$^{LiverOnly}$ mice with ezetimibe normalized biliary cholesterol levels and macrophage-to-feces RCT [122]. From these results, the authors concluded that biliary cholesterol secretion is needed for maximal macrophage-to-feces RCT, and that ezetimibe can promote RCT by inhibiting hepatic NPC1L1. We do not dispute the authors' conclusions, but do believe their data does not exclude the possibility that TICE is stimulated in NPC1L1$^{LiverTg}$ mice. Because of the lack of intestinal Npc1l1, all of the mice used in their study had significantly reduced cholesterol absorption and consequently much higher than normal fecal neutral sterol excretion. The NPC1L1$^{LiverOnly}$ mice displayed much greater mass and macrophage-derived fecal neutral sterol excretion compared to what we previously reported for NPC1L1$^{LiverTg}$ mice [122]. Therefore, in the face of a >80% decrease in biliary cholesterol concentration, it is likely that TICE contributed to the residual macrophage RCT observed in the NPC1L1$^{LiverOnly}$ mice. Given that NPC1L1$^{LiverOnly}$ have to rely solely on endogenous cholesterol synthesis for their total body cholesterol pool (i.e. cannot absorb dietary or bilary cholesterol), this model represents a pathophysiologic state where multiple pathways of cholesterol homeostasis are altered. Overall, it can be concluded that ezetimibe can promote RCT by blocking cholesterol absorption and inhibiting the reclamation of cholesterol from the bile by liver. In addition, ezetimibe can stimulate TICE but likely requires the hepatobiliary pathway to maximally stimulate RCT.

## THE UNEXPECTED ROLE OF HEPATIC FLAVIN MONOOXYGENASE 3 IN BALANCING BILIARY AND NON-BILARY RCT

Several lines of evidence suggest there is some mechanism of cross-talk between biliary and non-biliary pathways that synergize to maintain the set point of cholesterol excretion. For example, animals with genetic or surgical diversion of biliary cholesterol have normal or increased fecal cholesterol loss [9,11,33–44], indicating the liver was able to sense a defect in the biliary pathway and in a compensatory fashion upregulate TICE to maintain cholesterol balance. Recently, we set out to identify novel factors that regulate TICE and serendipitously uncovered a new pathway balancing both biliary and non-biliary RCT flux [123]. In an attempt to identify novel regulators of TICE we performed transcriptional profiling in NPC1L1$^{-LiverTg}$ mice (which exhibit chronic TICE stimulation [9]) and mice with hepatic knockdown of sterol o-acyltransferase (Soat2$^{HKD}$, which exhibit acute TICE stimulation [79]). From this screening, we found that the hepatic expression of flavin-containing monooxygenase 3 (FMO3) was coordinately downregulated in mouse models of stimulated TICE [123]. Several independent studies show that plasma levels of FMO3's enzymatic product trimethylamine-N-oxide (TMAO) are highly predictive of atherosclerosis in humans, and TMAO is proatherogenic in mice [124–130]. Given that we identified

alterations in the TMAO-producing enzyme FMO3 in TICE mouse models, and independent groups identified TMAO as a proatherogenic metabolite in humans, we initially took a loss-of-function approach to study the role of FMO3 in cholesterol metabolism and RCT [123]. In a TICE-like manner, ASO-mediated knockdown reduced biliary cholesterol secretion, while stimulating fecal neutral sterol loss [123]. Likewise, FMO3 knockdown promoted both basal and LXR-stimulated macrophage RCT, while reducing biliary cholesterol secretion [123]. Intriguingly, FMO3 knockdown closely phenocopies our preferred chronic TICE mouse model (NPC1L1$^{-LiverTg}$) [123], which displays severely reduced biliary cholesterol levels with normal fecal cholesterol loss [9,22]. It is also important to note that LXR activation alone significantly reduces hepatic FMO3 mRNA [123]. Collectively, our recent findings demonstrate that FMO3 gene expression is coordinately repressed in several mouse models of augmented TICE and that FMO3 knockdown promotes basal and LXR agonist-stimulated nonbiliary RCT [123]. However, it is important to point out that FMO3 knockdown alters multiple steps of cholesterol balance, making it difficult to know whether FMO3 is primarily affecting TICE or another component of cholesterol metabolism. We also saw deleterious effects of FMO3 including hepatic inflammation, further dampening enthusiasm for this potential RCT modulating target [123]. Overall, these recent observations identify the gut microbiota-driven FMO3/TMAO pathway as a key integrator of lipid metabolism, and specifically identify FMO3 as a novel regulator of sterol balance and RCT. Although these studies have identified FMO3 as a critical regulator of both biliary and non-biliary RCT pathways, targeting FMO3 is not likely a safe therapeutic strategy and would not specifically stimulate non-biliary RCT [123,124].

## Concluding remarks

The process of RCT has long been thought to play an atheroprotective role, yet recent failed clinical trials and Mendelian randomization studies have caused many to question the therapeutic benefit of HDL elevation. In the face of this growing doubt, there is little question that improving HDL function, facilitating removal of cholesterol from macrophage foam cells in the artery wall, and the excretion of cholesterol out of the body (all different steps in a common pathway) would provide therapeutic benefit to those suffering from CVD. Research focused on the RCT pathway during the last decade has been full of unexpected surprises, prompting substantial revision of the theoretical model of RCT flux. The new model includes classic centripetal flux back to the liver, but also includes additional steps where the liver determines whether to secrete cholesterol into bile or shunt a portion into the non-biliary TICE pathway. Undoubtedly, there are a number of outstanding questions that need to be addressed (Box 1). Although nearly all current RCT elevating strategies being tested in clinical trials are focused on HDL cholesterol elevation, there is no guarantee that raising HDL cholesterol will have a therapeutic benefit, given recent lessons learned. Moving forward it will be important to pay attention to the evolving RCT model, and design new therapies targeting specific branches of the RCT pathway that hold the most therapeutic promise for the patient. In particular, therapeutic strategies that specifically enhance the non-biliary TICE pathway hold immense untapped potential with no predicted side effects.

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## OUTSTANDING QUESTIONS BOX

In order to more fully understand therapeutic opportunities targeting the non-biliary TICE pathway (Figure 1), the following outstanding questions will need to be addressed:

1. Can we establish standardized quantitative methods to measure biliary and non-biliary fecal sterol loss in humans?
2. Are the apoB-containing lipoproteins that initiate TICE bona fide VLDL or a novel lipoprotein replete with its own proteome and cargo that allow for unique intravascular metabolism and recognition at the proximal small intestine?
3. What are the intestinal lipoprotein receptors/transporters that clear the hepatic apoB-containing lipoproteins that drive TICE?
4. What is the trafficking itinerary of TICE lipoproteins once they are taken up by intestinal enterocytes?
5. Can we identify drug targets to specifically modulate the intestinal component of RCT, while avoiding excessive biliary cholesterol secretion?

## HIGHLIGHTS

- Reverse cholesterol transport (RCT) involves both biliary and non-biliary pathways.
- The non-biliary pathway normally constitutes ~30% of RCT, but it can be stimulated.
- Flux through biliary and non-biliary RCT pathways is coordinated by the liver.
- Targeting the intestine as a cholesterol excretory organ holds therapeutic promise.

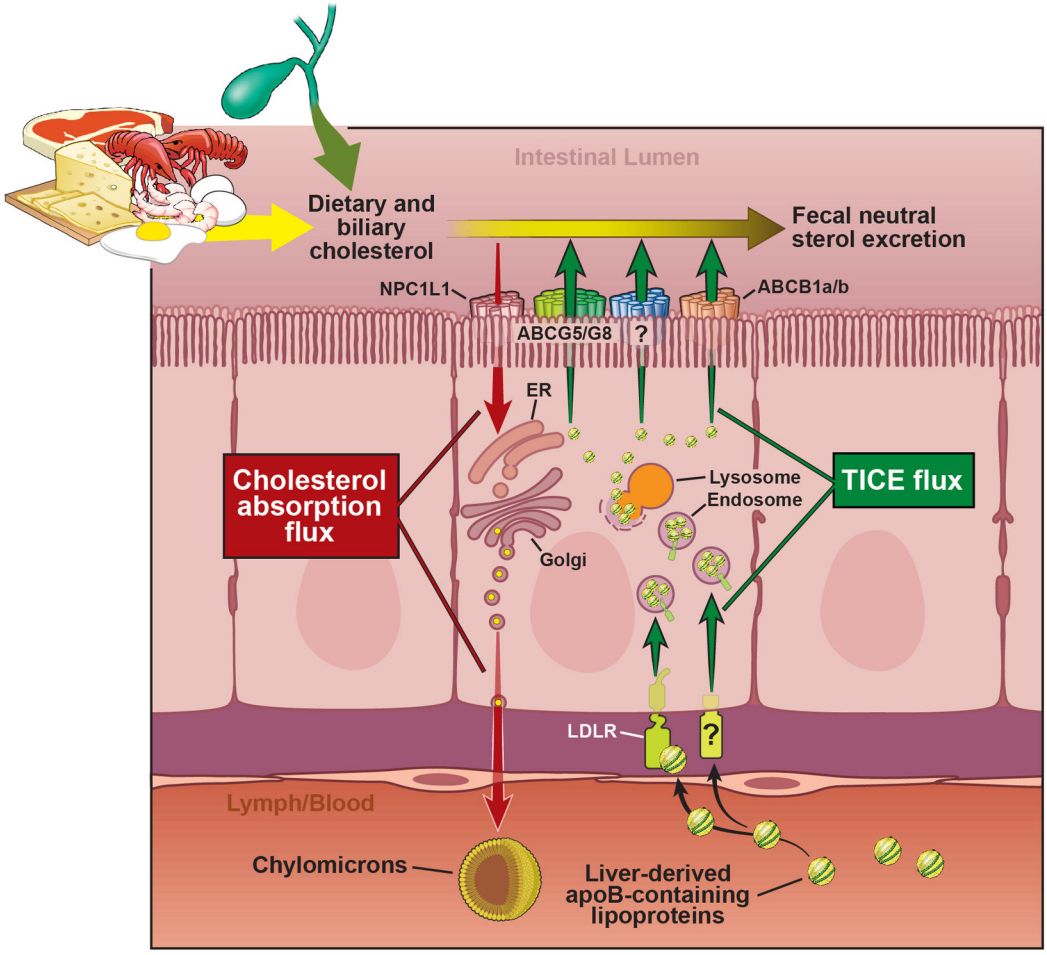


**Figure 1. Model for Integrated Biliary and Non-Biliary Reverse Cholesterol Transport**

Nascent HDL particles circulate to remove cholesterol from peripheral tissues and macrophage foam cells in the artery wall plaque. Once matured with both free and esterified cholesterol cargo, HDL is cleared by the liver primarily via SR-BI-dependent selective uptake. Importantly, in CETP-containing species, a large portion of HDL's cholesteryl ester cargo can be transferred to apoB-containing lipoproteins by CETP, which are taken up by the liver by hepatic low density lipoprotein receptors (LDLR) contributing to overall RCT flux. As shown by black arrows, all steps up to this point represent well understood pathways in RCT, and this shared centripetal flux collectively delivers cholesterol to the liver where this cargo then can branch into either biliary or less understood non-biliary pathways. As denoted with blue arrows, a large portion of the HDL-derived cholesterol pool can be secreted into bile via the actions of proteins such as ATP-binding cassette transporters G5 and G8 and other minor mechanisms. Once cholesterol is secreted into bile a large portion of this pool is physically delivered to the lumen of the small intestine via the common bile duct, where it can ultimately provide substrate for fecal cholesterol loss. Alternatively, highlighted by red arrows, the liver can initiate the non-biliary TICE pathway to eliminate excess cholesterol. Current evidence suggests that the non-biliary branch of RCT can be initiated by either re-uptake of biliary cholesterol via the cannalicular sterol

transporter NPC1L1 among other mechanisms. Following NPC1L1-dependent recovery of biliary cholesterol, the excess free cholesterol is moved to the ER where it is repackaged onto nascent apoB-containing lipoproteins, which are ultimately secreted from the liver into the bloodstream. The liver-derived apoB-containing lipoproteins are then recognized by the proximal small intestine through lipoprotein receptors such as LDLr, and likely other mechanisms. Once cleared by the proximal small intestine, TICE-derived cholesterol is directionally trafficked across the enterocyte in a basolateral to apical fashion, and this cholesterol can be effluxed across the apical membrane via the actions of ATP binding cassette transporters ABCG5/ABCG8, ABCB1a/b, and likely other mechanisms. Collectively this TICE flux through the intestine, coupled with biliary cholesterol secretion, and dietary cholesterol make up the sum total of cholesterol available for excretion into the feces. New evidence suggests that the hepatic enzyme flavin containing monooxygenase 3 add another level of control, functioning to balance RCT flux by enhancing the biliary pathway and suppressing the TICE pathway. Abbreviations used: ABCB1a/b = ATP-binding cassette transporter 1a/b; ABCG5/G8 = ATP-binding cassette transporters G5 and G8; ACAT2 = acyl-CoA:cholesterol acyltransferase 2; apoB = apolipoprotein B; CE = cholesteryl ester; ER = endoplasmic reticulum; FC = free cholesterol; FMO3 = flavin containing monooxygenase 3; HDL = high density lipoprotein; LDL = low density lipoprotein; LDLR = low density lipoprotein receptor; MTP = microsomal triglyceride transfer protein; NPC1L1 = Niemann-Pick C1-like 1; PCSK9 = proprotein convertase subtilisin/kexin type 9; SR-BI = scavenger receptor class B class I; TICE = transintestinal cholesterol excretion; ? = unknown proteins.

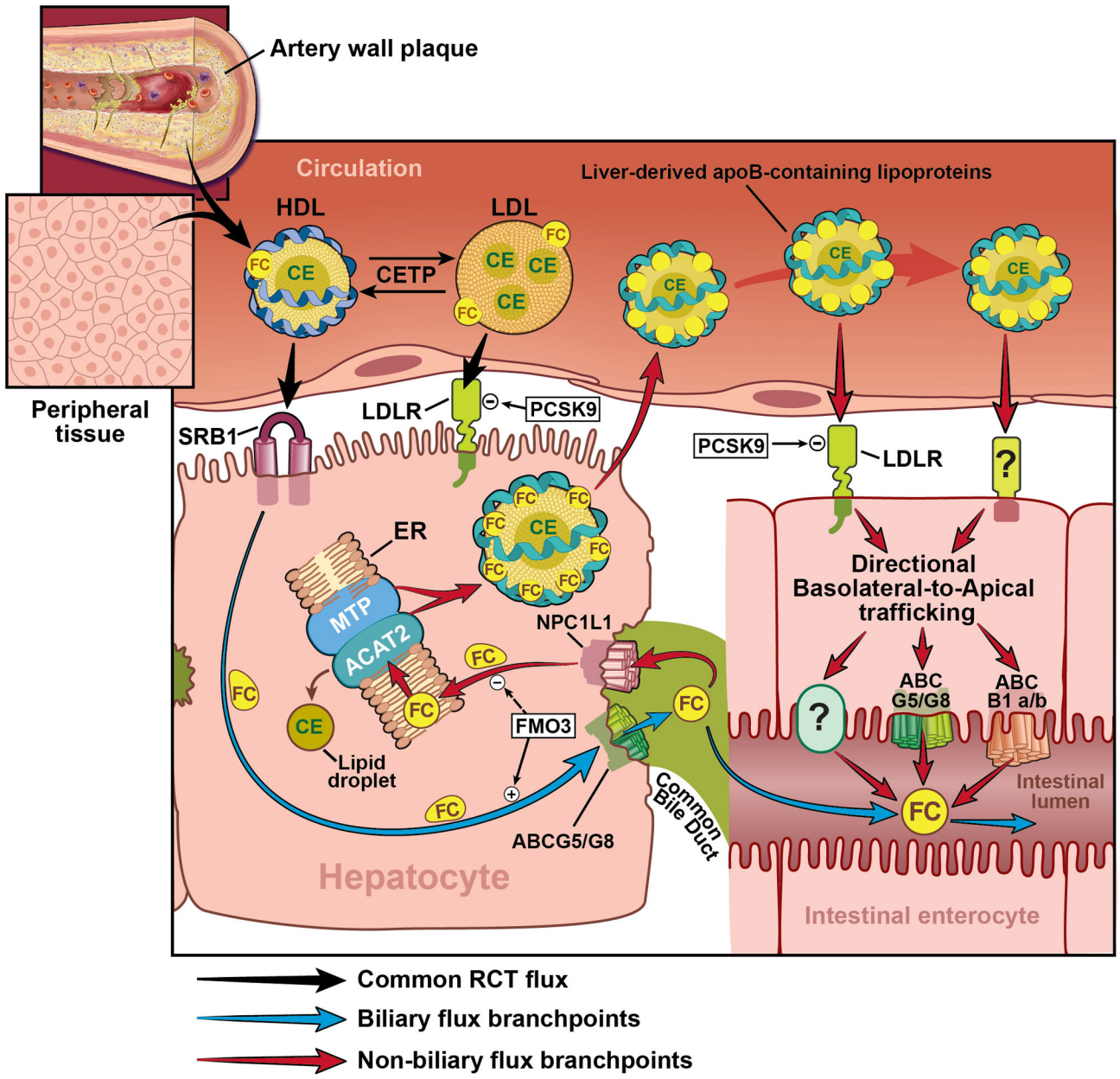


**Figure 2. Model for Cholesterol Flux Across the Intestinal Enterocyte**

The intestinal enterocyte is a gatekeeper of cholesterol balance with two opposing pathways delivering cholesterol from opposite sides of this polarized cell. First, the cholesterol absorption pathway is initiated by NPC1L1-dependent delivery of dietary and biliary cholesterol to the ER, where it is packaged into nascent chylomicron particles for delivery into the lymphatics. Directly opposing the absorptive pathway, TICE flux is driven by the delivery of liver-derived apoB-containing lipoproteins that are taken up by the LDL receptor and likely other receptors. Once internalized these lipoproteins are trafficked through endosomal and lysosomal compartments, and ultimately effluxed from the apical membrane through ABCG5/ABCG8, ABCB1a/b, and likely other transporter. A portion of this newly

effluxed cholesterol can be excreted into the feces. Collectively, these two opposing influx pathways (absorption and TICE) along with endogenous cholesterol synthesis comprise the total enterocyte cholesterol pool. Abbreviations used: ABCB1a/b = ATP-binding cassette transporter 1a/b; ABCG5/G8 = ATP-binding cassette transporters G5 and G8; apoB = apolipoprotein B; ER = endoplasmic reticulum; LDLR = low density lipoprotein receptor; NPC1L1 = Niemann-Pick C1-like 1; TICE = transintestinal cholesterol excretion; ? = unknown receptor mediating the uptake of TICE lipoproteins.