Novel insights in cholesterol excretion
van der Velde, A.E.

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DISCUSSION, CONCLUSIONS, AND PERSPECTIVES
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This thesis primarily deals with the re-discovery of a “forgotten” cholesterol excretion pathway: transintestinal cholesterol efflux (TICE). In mice this appears to be one of the last steps in a pathway called reverse cholesterol transport (RCT). A start was made to unravel the underlying mechanisms behind this process. The first step in RCT is export of cholesterol form peripheral cells to an acceptor in plasma.

In chapter 2 the stimulating effect of a novel drug called fatty acid-bile acid conjugate (FABAC) on the first step of RCT was described. Aramchol (the most potent FABAC dose dependently increased cholesterol efflux from human skin fibroblasts in the absence of known efflux mediators such as apoA-I. Interestingly, the stimulatory effect of aramchol on cholesterol efflux depended on the activity of ABCA1.

Chapter 3 focuses on one of the last steps of cholesterol removal: the secretion of cholesterol by the intestine. In chapter 3 we show that, using an intestinal perfusion set-up, significant amounts of cholesterol were secreted via the intestine in mice. These results were confirmed by cholesterol balance studies. In mice, the amount of cholesterol secreted via the intestine is approximately twice the amount secreted via the hepatobiliary pathway (for example: in FVB mice 5.4 ± 1.8 versus 1.9 ± 0.8 μmol/day.100 g body wt). The majority of transintestinal cholesterol efflux takes place in the proximal small intestine.

**Origin of intestinally secreted cholesterol**

Kruit et al. (1) showed in Abcb4/- mice that intravenously injected free cholesterol partly ended up in the feces as neutral sterol. Their experiments were performed in Abcb4/- mice with abrogated biliary phospholipid and cholesterol secretion thus providing evidence, at least in these mice, for a direct route from blood to intestinal lumen. Similar results were obtained in wild-type mice, suggesting that also in wild-type animals intestinally secreted cholesterol originated from blood (chapter 3). Assuming that the major part of the secreted cholesterol directly came from the blood compartment bypassing the liver, the next obvious question is which pathways are involved in this process. Uptake via lipoproteins followed by endosomal/lysosomal processing would lead to equilibration of plasma cholesterol with the enterocyte cholesterol pool. To establish whether this is a likely process for
TICE, we measured the specific radioactivity of secreted cholesterol in perfusate and in blood and mucosa. If this pathway is likely to occur, the specific radioactivity of cholesterol should be similar to the specific radioactivity of the cholesterol pool in enterocytes. This was indeed observed (chapter 3). The specific radioactivity in serum was 10 fold higher. This indicates that TICE is not a fully paracellular pathway. Since equilibration of cholesterol with the enterocyte cholesterol pool should take place before excretion in the intestinal lumen, the sterols excreted directly via the enterocytes could, in principle, also originate from shed cells. To establish the contribution of cell shedding to luminal cholesterol we measured a variety of cytosolic and apical markers in mucosa and perfusate, based on those measurements we concluded that cell shedding plays a minor role as source for intestinally secreted cholesterol (chapter 3). Cholesterol can be synthesized in virtually every tissue, however, most tissues make a minor contribution to total synthesis. The most striking difference among different species in tissue sterol synthesis rate is observed in the liver which accounts for, for example, 51 % of the newly synthesized sterol in the rat but for only 16 % in guinea pig. Of the remaining tissues, 3 contributes to cholesterol synthesis in all species: the gastrointestinal tract, skin, and carcass. Furthermore, in those species in which the liver makes a relatively small contribution to total body sterol synthesis, these 3 tissues become quantitatively much more important as sites for sterol synthesis. The upper gastrointestinal tract (stomach, jejunum, and ileum), for example, contains about 13 % of the newly synthesized sterol in the rat but 27 % in the guinea pig (2). The small intestine plays a prominent role in cholesterol synthesis in mice as well (3). To examine whether cholesterol synthesized in enterocytes could be an important source of intestinally secreted cholesterol, mRNA levels of Hmg-CoA reductase in intestines of mice that were perfused without a cholesterol acceptor (low TICE) were compared to Hmg-CoA reductase mRNA levels in intestines of mice which were perfused with TC/PC micelles (relatively high TICE). No correlation between Hmg-CoA reductase expression and TICE was observed (chapter 4). Van der Veen et al. (4) developed a method to quantify the fractional and absolute contributions of several cholesterol fluxes to total fecal neutral sterol loss in vivo in mice. Confirming our observations, they established that TICE is an important pathway for cholesterol removal in mice. Furthermore, they demonstrated that cholesterol secreted via the intestine primarily originates from blood rather than from the intestine itself. In summary, the body is capable of secreting significant
amounts of cholesterol via the intestine. Cholesterol in blood is taken up by enterocytes and equilibrates with the enterocyte cholesterol pool before it is secreted.

**The process underlying TICE**

TICE is a specific process and probably involves activity of transport proteins on both basolateral and apical sides of the enterocytes. The role of several putative transporters involved in TICE was investigated.

Sr-b1 is known to mediate cholesterol uptake from circulating HDL and LDL (5; 6), although, HDL appeared to be a more effective donor (6). In Caco-2 cells, SR-B1 expression is observed at basolateral, as well as, on apical membranes (7). This suggests that Sr-b1 has the potential to serve several functions in the intestine. The localization of Sr-b1 on the basolateral surface of the intestine suggests its possible involvement in intestinal lipoprotein uptake (7). We showed that mice receiving a Western-type diet (0.25 % (w/w) cholesterol) or a high fat diet (no cholesterol), have increased TICE. Intestinal Sr-B1 mRNA, as well, as protein levels, correlated to the rate of TICE. Therefore, the role of Sr-B1 in TICE was evaluated. Unexpectedly, TICE was significantly increased in Sr-B1 deficient mice (chapter 4). The role of HDL as appreciated cholesterol donor for the intestine is questionable. Abca1-/- mice, mice with very low HDL levels, for example, do not show a diminished fecal neutral sterol output compared to their littermates (8).

The sterol efflux proteins ABCG5/ABCG8 seem to be good candidates for the mediation of cholesterol secretion from the enterocyte into the intestinal lumen. Transgenic animals overexpressing human ABCG5 and ABCG8 (hG5G8Tg) showed a severely increased fecal neutral sterol excretion (9). Furthermore, Van der Veen et al. (4) showed that the TICE flux was decreased in Abcg5-/- mice causing a reduction in total fecal neutral sterol excretion. On the other hand, the increased fecal neutral sterol excretion in the hG5G8Tg mice was inhibited in hG5G8Tg mice lacking Mdr2, this suggests that biliary excretion is responsible for the increased fecal neutral sterol excretion in hG5G8Tg mice (10). Furthermore, deficiency of Abcg5 and/or Abcg8 normally leads to mild (11; 12) or no (13) decrease in fecal neutral sterol excretion and the mRNA expression and protein levels of Abcg5/Abcg8 (14) throughout the intestine does not correlate with the rate of TICE in the same intestinal segment. Moreover, no reduction in TICE was
Discussion, conclusions, and perspectives

measured in Abcg8-/- mice using the intestinal perfusion set-up (chapter 3). In summary, the role of Abcg5/Abcg8 in TICE remains controversial.

To discover putative candidate proteins involved in TICE, gene expression analysis of proteins known to play a role in cholesterol trafficking elsewhere in the body was performed with the intestines of perfused mice (chapter 4 and 5). For this gene expression analysis, the intestines of non-treated wild-type mice and mice that displayed increased TICE such as Western-type or high fat diet fed mice and mice that received a PPARδ agonist, were used to observe whether a correlation existed between the rate of TICE and the intestinal expression of the investigated genes. Besides Abcg5/Abcg8 and Sr-b1, no apparent involvement of proteins involved in lipoprotein metabolism: MTP, LRP, LDLR, VLDLR (data not shown); or the ABC transporters: Abca1 and Abcg1; could be established (chapter 4 and 5). Some proteins involved in intracellular trafficking were also evaluated. Cholesterol metabolism is compartmentalized into distinct subcellular membranes. Cholesterol reaches the plasma membrane mostly via Golgi bypass route(s). Lipoprotein cholesterol is internalized via receptor-mediated uptake and reaches the endocytic circuits from where it is redistributed to the plasma membrane, Golgi complex, and ER. Trafficking involves multiple proteins among them Rab proteins (see for review: (15)). Interestingly an increase in TICE went hand in hand with a pronounced increase in expression of Rab9 protein (chapter 5). Rab9 is a key late endosomal small GTPase. Overexpression of Rab9 in Niemann-Pick type C lipid storage disease cells results in correction of lipid trafficking defects like restoration of Golgi targeting of glycosphingolipids (16; 17) and dramatic reduction of intracellular cholesterol storage (16-19). Rab9 is not only able to lower cholesterol storage in Niemann-Pick type C diseased cells but also in cells of cystic fibrosis patients when Rab9 is overexpressed (20). Besides Rab9 expression, the expression of another protein, possibly involved in intracellular cholesterol trafficking: lysosomal integral membrane protein 2 (LIMP-2) was increased. The exact role of intracellular cholesterol trafficking as part of TICE needs to be elucidated in more detail.

With regards to stimulation of TICE, it is interesting to mention that arachachol increased fecal sterol output in rats without changes in biliary lipid output (21). These results made arachachol an interesting candidate for TICE stimulation. However, a pilot experiment investigating the effect of arachachol on TICE did not reveal a stimulatory effect of arachachol on TICE. Since fecal neutral sterol output of
the aramchol treated mice also only mildly increased, the effect of aramchol on TICE remained unclear. Investigation of the effect of aramchol on TICE in more detail would be interesting.

**The human situation**

Fecal sterols of non-dietary origin, are present in feces of patients with biliary obstruction (22). Assuming that intestinal cell shedding is not a prominent contributor to fecal sterols, this suggests that TICE is present in these patients. Question remains whether TICE is present under basal conditions in humans. In 1967 Simmonds et al. (23) performed elegant intestinal perfusion studies in humans and observed significant secretion of cholesterol in the small intestine. However, the amount of secreted cholesterol was not quantified. On average, humans secrete about 1 gram of neutral sterols per day (24; 25). Dietary cholesterol intake is about 400 mg (26) and biliary cholesterol secretion amounts to 1000 mg (27-29). Cholesterol absorption has been estimated to be about 50 % (24; 25; 30). Hence, the average direct intestinal secretion in humans can be estimated to be around 300 mg/day.70 kg body weight, which is about one-third of the amount secreted into bile. The difference between mice and human in contribution of TICE to total cholesterol excretion might be explained by the fact that mice have a relatively larger surface area of small intestinal epithelium in comparison to human. If TICE is observed throughout the small intestine, it might be expected that species that have a relatively larger surface area secrete relatively more cholesterol. For most species there is an inverse relation between the gross surface area of the small intestine and body size. The ratio intestinal surface area/body size is related to the diet eaten by each species (31). Carnivores possess a short intestine, while herbivores and other non-meat eating species, like mice, need a relatively long intestine because the digestion of their food takes more time. The length of the intestine of omnivores, like man, is in between that of carnivores and herbivores (32).

**Comparison of the regulation of TICE in man versus mice**

TICE was twofold elevated in mice receiving a Western-type diet or a high fat-only diet. This increase correlated with a twofold increase in neutral sterol output found in high fat diet fed mice. A high cholesterol-only diet did not cause an increase in TICE in mice. The lack of effect of the high cholesterol diet despite higher biliary
cholesterol secretion strongly suggests that the rate of TICE is mainly controlled by the fat content of the diet (chapter 4). What about the effect of dietary fat on sterol excretion in humans? In the late fifties/early sixties of the last century, the effect of dietary fat on plasma cholesterol levels and a possible relationship between serum cholesterol levels and fecal sterol excretion were intensively investigated in two laboratories. In 10 out of 12 patients studied, plasma cholesterol changes were observed without changes in excretion (33-35). These results implicate that changes in fecal neutral sterol excretion cannot be fully explained by changes in plasma cholesterol levels and that changes in dietary fat did not affect sterol excretion in a consistent manner. However, the subjects participating in these studies were mostly hypercholesterolemic or hypertriglyceridemic individuals with xanthomatous disease in whom cholesterol storage in the skin, tendons, and perhaps other tissues was present. When Connor et al. (36) investigated the effect of dietary fat on fecal sterol excretion they recruited men with characteristic serum cholesterol concentrations for the American population at their age and men without known metabolic disorders. In this study, polyunsaturated fats did affect fecal sterol excretion. Interestingly, the hypocholesterolemic effect of polyunsaturated fat was associated with total fecal sterol excretion twice greater than the amount of cholesterol calculated to leave the plasma. This not only suggests, that polyunsaturated fat increases fecal sterol excretion in normal, healthy subjects, but that this also might be caused by an increase in TICE. The effect of dietary polyunsaturated fat on fecal neutral sterol excretion in normal men was reported also in later studies by Nestel et al. (37) and Oh et al. (38). In all these studies the consumption of saturated fat enhanced fecal neutral sterol excretion but to a lesser extent than polyunsaturated fat (36-38). So whereas the increase of fecal output of endogenous steroids has been recorded by many investigators, results on the output of endogenous neutral steroids in hypertriglyceridaemic patients remains contradictory. Simons and Myant (39) suggested that the conflicting observations in hypertriglyceridaemic patients might be explained by the type of hyperlipoproteinaemia.

In chapter 4 we investigated the effect of luminal modifications on TICE in mice by using different bile salts and/or phospholipids. The rate of TICE appeared to be remarkably insensitive to the type of bile salt and intraluminal bile salt concentration. Neither the luminal bile salt concentration, nor bile salt hydrophobicity affected TICE, indicating that the presence of phospholipids is the
dominant factor modulating the amount of cholesterol secreted via TICE. As shown in chapter 3, bile salts alone also stimulate TICE, hence the presence of phospholipids is not essential. This conclusion is supported by the fact that Abcb4⁻/⁻ mice with very low luminal phospholipids levels show unaltered fecal neutral sterol excretion suggesting that TICE is not negatively affected in these mice (1). The stimulating effect of phospholipids on TICE may explain in part why fecal sterol excretion is strongly stimulated by phospholipid supplementation to the diet of human (40).

PPARδ activation leads to increased cholesterol efflux from macrophages (41-43), without affecting biliary cholesterol secretion rates (43). In chapter 5, we showed, in mice, that PPARδ activation stimulated TICE. PPARδ activation has been shown to accelerate RCT, not only in mice (43) but also in primates (41) and man (42). It is tempting to hypothesize that PPARδ activation will stimulate TICE in man as well.

So although the relative contribution of TICE apparently is smaller in humans than in mice, the magnitude of this flux is still considerable and activation of this pathway seems an attractive approach for treatment/prevention of atherosclerotic diseases.
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