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## The ABC of cholesterol transport

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# CHAPTER 7

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## The ABC of hepatic and intestinal cholesterol transport

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## Summary

Liver and (small) intestine are key organs in maintenance of cholesterol homeostasis: both organs show active *de novo* cholesterologenesis and are able to transport impressive amounts of newly synthesized and diet-derived cholesterol via a number of distinct pathways. Cholesterol trafficking involves the concerted action of a number of transporter proteins, some of which have been identified only recently. In particular several ATP-binding cassette (ABC) transporters fulfill critical roles. For instance, the ABCG5/ABCG8 couple is crucial for hepatobiliary and intestinal cholesterol excretion, while ABCA1 is essential for HDL formation and, hence, for interorgan trafficking of the highly water-insoluble cholesterol molecules. Very recently, the Niemann-Pick C1 Like 1 protein has been identified as a key player in cholesterol absorption by the small intestine and may represent a target of the cholesterol absorption inhibitor ezetimibe. Alterations in hepatic and intestinal cholesterol transport affect circulating levels of atherogenic lipoproteins and thus the risk for cardiovascular disease. This review specifically deals with the processes of hepatobiliary cholesterol excretion and intestinal cholesterol absorption as well as the interactions between these important transport routes. During the last few years, insight in the mechanisms of hepatic and intestinal cholesterol transport has greatly increased not in the least by the identification of involved transporter proteins and the (partial) elucidation of their mode of action. In addition, information has become available on (transcription) factors regulating expression of the encoding genes. This knowledge is of great importance for the development of a tailored design of novel plasma cholesterol-lowering strategies.

## **Introduction**

Elevated plasma cholesterol concentrations comprise a major risk factor for development of atherosclerosis and cardiovascular disease remaining the leading cause of morbidity and mortality in Western societies. This, in combination with the fact that a relatively high proportion of hypercholesterolaemic patients fail to reach their target LDL-cholesterol concentrations on “standard” (diet, statins) therapy alone, provides the basis for a quest for more effective treatment modalities and/or supportive strategies. The development of ezetimibe, a specific and potent inhibitor of intestinal cholesterol absorption<sup>1</sup> that reduces plasma LDL-cholesterol by ~20% in mildly hypercholesterolemic patients,<sup>2</sup> and the established LDL-lowering effects of dietary plant sterols/stanols<sup>3</sup> that interfere with cholesterol absorption has focused attention to the intestine as a promising site of action. As a consequence, there is an increased interest in achieving a better understanding of the molecular mechanisms involved in control of intestinal cholesterol absorption. Uptake from the intestine represents a major source for cholesterol entry into the body pools.<sup>4</sup> However, it should be realized that although the intestine is an important station in cholesterol trafficking, the liver is the dominant regulatory unit. Therefore, the plasma cholesterol-lowering effects of cholesterol absorption inhibitors are primarily brought about by metabolic adaptations in the liver, *i.e.*, the organ in which diet-derived cholesterol ends up via the chylomicron remnant pathway. Furthermore, it must be kept in mind that the major part of cholesterol that is taken up by the intestine on a daily basis is not derived from the diet but is actually biliary cholesterol that comes directly from the liver. Therefore, to be able to design more effective strategies for prevention or treatment of cardiovascular disease a comprehensive and integrated picture of intestinal and hepatic cholesterol metabolism is required. During the last few years, there have been highly significant advances in our understanding of specific areas of cholesterol transport, particularly concerning mechanisms of hepatobiliary cholesterol excretion and the actual cholesterol absorption process. The current chapter reviews these recent developments and addresses some of the still unresolved issues.

## **Quantitative estimates of cholesterol transport rates**

The total turnover of body cholesterol pools in adult humans (~70 kg) equals about 1-1.5 grams per day, *i.e.*, in the order of 1% of the whole body cholesterol content.<sup>4</sup> A major part of this turnover reflects the conversion of cholesterol to bile salts by the liver and their subsequent loss via the feces.<sup>5</sup> Under steady state conditions, the loss of cholesterol from the body is compensated for by *de novo* synthesis and absorption from the diet. *De novo* synthesis in adults amounts up to 0.6-1 g/day, as revealed by careful balance studies and confirmed by

direct measurements employing stable isotope techniques (*e.g.*, Neese *et al.*<sup>6</sup>). A “typical Western diet” provides 0.3-0.5 g/day of cholesterol which mixes with an even larger amount of biliary cholesterol, *i.e.*, ~ 1 g/day, in the upper small intestine. There are data to indicate that biliary cholesterol is absorbed from the intestine with greater efficacy than dietary cholesterol is,<sup>7</sup> because it is delivered in mixed micelles and therefore readily available for absorption. Cholesterol therefore undergoes extensive enterohepatic circulation, which represents an important yet often ignored factor in the control of cholesterol homeostasis. In view of the fact that most studies show that humans absorb about 50% of all cholesterol entering the intestine (*e.g.*, Ostlund *et al.*<sup>8</sup>), it is clear from these figures that the intestine processes a considerable amount of cholesterol each day, which, via the chylomicron remnant pathway, is directly delivered to the liver. Because the liver is the principal site for the production as well as the clearance of LDL-cholesterol,<sup>9</sup> alterations in the delivery of intestine-derived cholesterol to the liver can potentially have a significant impact on plasma LDL-cholesterol concentrations through interference with hepatocytic cholesterol metabolism.

The massive enterohepatic circulation of cholesterol has important but often underestimated methodological implications when evaluating cholesterol absorption in experimental settings. In most recent studies in humans as well as in experimental animals, dual (radioactive or stable) isotope tracer techniques are used to estimate fractional cholesterol absorption. Principally, two approaches can be discerned, *i.e.*, the dual-isotope fecal collection method and the dual-isotope plasma ratio method. In the first, labeled cholesterol is given orally together with a labeled non-absorbable marker, in most cases sitostanol or sitosterol, and feces is collected for a given period of time. The fecal ratio of labeled cholesterol over marker provides an estimate of the fractional absorption rate. The second method requires simultaneous administration of exact amounts of (differently) labeled cholesterol both intravenously and orally/intragastrically, and plasma ratio's over time provide a value for the fractional absorption rate. The pro's and con's of both methods have recently been discussed by Turley and Dietschy<sup>4</sup> and Wang and Carey<sup>10</sup>: the important issue with respect to interpretation of the data is that the cholesterol absorption rates obtained always reflect a fractional (percent) value and are by definition not a measure of the absolute amount of cholesterol that is delivered to the liver from the intestine. For the latter, one needs to know the mass of the intraluminal cholesterol pool and this is usually not the case since the contribution from bile and other endogenous sources (*e.g.*, sloughing of intestinal cells) is unknown in most situations. Hence, a direct translation of changes in fractional absorption rates to absolute changes in the amounts of chylomicron remnant cholesterol reaching the liver is sometimes difficult to make.

## **Mechanisms of hepatobiliary cholesterol transport**

Bile formation is an important function of the liver, which is performed by the parenchymal liver cells or hepatocytes. Hepatocytes are polarized cells with their basolateral (sinusoidal) membrane facing the blood and their apical (canalicular) membrane facing the bile canaliculus. Both membrane domains are separated from each other by tight junctions. Bile is an aqueous solution that contains, apart from a variety of other organic molecules, bile salts, phospholipids and free cholesterol in millimolar concentrations. These bile components are mainly present in the form of aggregates, *i.e.*, mixed micelles, simple micelles or vesicular structures (see Verkade *et al.*<sup>11</sup>). Formation of bile is an osmotic process. Bile salt secretion, which is mediated by the so-called Bile Salt Export Pump (BSEP) or ABCB11, provides the major driving force for bile formation and, in addition, stimulates the secretion of cholesterol (see below).

## **Dependency of biliary cholesterol secretion on bile salt and phospholipid secretion**

It has been known for decades that biliary cholesterol and phospholipid secretion is tightly coupled to that of bile salts. Infusion of bile salts into different animal species as well as in humans invariably leads to induction of biliary cholesterol (and phospholipid) secretion (see Verkade *et al.*<sup>11</sup> for review). The stimulatory actions of bile salts on biliary lipid secretion depend to a large extent on their relative hydrophobicity: the more hydrophobic the higher their efficacy to induce lipid secretion. When bile salts are incubated with isolated cells or erythrocytes, release of cholesterol and phospholipid is readily induced.<sup>12,13</sup> Hence, biliary lipid secretion that occurs at the canalicular pole of the hepatocyte could be a passive process fully controlled by the detergent actions of bile salts. It therefore came as a great surprise when it was found that biliary lipid secretion is fully abrogated in mice lacking the gene encoding mdr2 P-glycoprotein (*Mdr2*), now known as *Abcb4*.<sup>14</sup> This ABC transporter supposedly mediates transport of phosphatidylcholine from the inner leaflet to the external leaflet of the canalicular membrane where, in concert with bile salts, phospholipid/cholesterol-containing vesicles are formed that are subsequently secreted into the canalicular lumen. The most convincing evidence for this concept came from studies of the group of Crawford<sup>15-17</sup> demonstrating the presence of vesicle-like structures expanding from the canalicular membrane using ultra-rapid fixation of liver tissue. As far as we know, reconstitution of this system in cultured polarized cell systems has not succeeded. Therefore, formal proof of this mechanism of lipid secretion is still lacking. ABCB4 is supposed to provide the driving force for vesicle formation since in the absence of this protein no vesicles

could be detected.<sup>17</sup> Since the *Abcb4*-null mice, in addition to a complete absence of phospholipid, also show a virtually complete absence of biliary cholesterol secretion,<sup>14,18,19</sup> the sterol was supposed to follow phospholipids passively. However, this concept has appeared to be too simplistic, because in later studies it was shown that cholesterol secretion can be restored to almost normal levels when *Abcb4*-null mice are infused with hydrophobic bile salts.<sup>19</sup> A clear uncoupling of cholesterol secretion into bile from that of phospholipids and bile salts has also been shown in a number of other conditions.<sup>11</sup> Biliary cholesterol secretion shows great species-to-species variation whereas the cholesterol content in the liver seems much less variable.<sup>20</sup>

### **The *Abcg5/Abcg8* heterodimer as mediator of biliary cholesterol secretion**

Very recently, candidate proteins that may account for the phenomena described in section 1.2.1 have been identified. In 2002, the groups of Hobbs<sup>21</sup> and Patel<sup>22</sup> almost simultaneously identified mutations in the genes that encode two ABC halftransporters, *i.e.*, *ABCG5* and *ABCG8*, that underlie the inborn error of metabolism called sitosterolemia. Patients suffering from this disease accumulate large amounts of plant sterols in their bodies, have an increased cholesterol absorption, a decreased bile cholesterol secretion, and, finally, a complete abrogation of secretion of plant sterols into the bile.<sup>23-25</sup> *ABCG5* and *G8* were postulated to function as exporters of sterols in the form of a heterodimer at the apical membranes of small intestinal epithelial cells (see below) and of hepatocytes. Defects in either of the two proteins is sufficient to cause the full phenotype of sitosterolemia, suggesting that expression of only one of the two proteins does not salvage the transport function.

*ABCG5/Abcg5* and *ABCG8/Abcg8* are predominantly expressed in hepatocytes and in small intestinal enterocytes in humans and mice. The two genes are arranged in a head-to-head configuration in the human<sup>26</sup> and mouse<sup>27</sup> genome. Expression of both genes is coordinately regulated and highly induced in mice kept on a high-cholesterol diet.<sup>28,29</sup> *LXR $\alpha$* , a nuclear receptor activated by oxysterols that plays a crucial role in regulating genes involved in cholesterol trafficking, is required for induction of murine *Abcg5* and *Abcg8* expression upon cholesterol feeding.<sup>28</sup> Treatment of mice with synthetic *LXR* agonists induces the expression of both genes in liver and intestine.<sup>28,30</sup>

Via adenoviral overexpression of the human genes in cell lines it was recently found that both proteins are required to ensure proper processing from ER through Golgi and subsequently to the apical membrane. *ABCG5* or *G8* expressed singly remained in the ER. These studies by Graf *et al.*<sup>31</sup> were carried out with tagged proteins which still leaves the possibility open that the tags may have disrupted the normal routing of the proteins. The proposed concept that simultaneous expression of *ABCG5* and *G8* is at least essential for proper

transport via the secretory pathway could explain why mutations in either gene induce the full phenotype in sitosterolemia patients. The group of Hobbs has subsequently constructed transgenic *ABCG5/G8* overexpressing mice as well as double knock-out mice.<sup>32,33</sup> In the transgenics, a P1 clone containing both human genes with the connecting promotor region was inserted, leading to up to fourteen times overexpression of both genes exclusively in liver and intestine.<sup>32</sup> In gallbladder bile of these mice, cholesterol content was five-fold increased while bile salt content was unchanged. This supported an important role of the heterodimer in biliary cholesterol secretion. Detailed analysis of *Abcg5/g8* double knock-out mice confirmed the role of the heterodimer in biliary sterol secretion.<sup>33</sup> The cholesterol content of gallbladder bile was decreased by more than 90% in these mice whereas no significant effects on bile salt content were observed. Interestingly, also plasma and liver cholesterol contents were decreased in the *Abcg5/g8* knock-out mice. Since the content of plant sterols was increased dramatically, secondary effects caused by these sterols may underlie this phenomenon. Heterozygous *Abcg5/g8* mice showed a 30-40% decrease in biliary cholesterol concentration, indicating that the biliary phenotype is not due to a secondary effect induced by massive amounts of plant sterols in the liver. Feeding these mice a high-cholesterol diet induced a massive increase of cholesterol in the liver but had little effect on biliary cholesterol secretion, again indicating a crucial role of ABCG5 and ABCG8 in biliary cholesterol secretion.<sup>33</sup>

The molecular mechanism by which the ABCG5/ABCG8 heterodimer mediates cholesterol secretion is still an enigma. It is generally assumed that cholesterol readily flips between the lipid bilayers in biological membranes. Studies in model membranes invariably show high rates of flipping, which making a role for ABCG5/G8 as a cholesterol flippase unlikely. Recently, Small<sup>34</sup> advanced an alternative hypothesis. In his view, ABCG5/G8 activity decreases the activation energy for cholesterol efflux out of the outer leaflet of the canalicular membrane. If Small's hypothesis would be true, current schemes of the mechanism of biliary cholesterol secretion need substantial revision. Presently, co-secretion of the two lipids in the form of vesicles is the most favored mechanism, based on the rather strict coupling of cholesterol and phospholipid that has been observed in a large variety of studies. However, when ABCG5 and G8 indeed serve to lower activation energy for cholesterol efflux, one might assume that mixed micelles would be the preferred carrier to capture this activated cholesterol rather than to assume that cholesterol diffuses laterally into phospholipid domains before their combined secretion in the form of vesicles.

Based on the genetics of sitosterolemia, *i.e.*, no phenotypic differences in humans with defects in either *ABCG5* or *ABCG8*, mouse studies and *in vitro* studies as described above, it was hypothesized that *Abcg5* and *Abcg8* function as obligate heterodimers. In line herewith, QTL analysis identified *Abcg5/Abcg8* as important genes for the genetic susceptibility and pathogenesis of cholesterol cholelithiasis in inbred strains of mice.<sup>35</sup> Kusters *et al.*<sup>36</sup> found a close relationship between biliary cholesterol secretion rates normalized to phospholipid secretion and both hepatic *Abcg5* and *Abcg8* expression levels normalized to *Abcb4*

expression, when various mouse models of cholesterol hypo- and hypersecretion were included in the analysis. It should be noted, however, that there was one exception to the rule: the 15-fold induction of biliary cholesterol secretion induced by diosgenin feeding occurred without any change in *Abcg5/Abcg8* expression. Very recently, Klett *et al.*<sup>37</sup> reported a mouse model of sitosterolemia created by a targeted disruption of the *Abcg8* gene alone. These mice showed very significantly elevated levels of plasma and tissue plant sterols (sitosterol, campesterol) consistent with sitosterolemia. These mice also showed an impaired ability to secrete cholesterol into bile (-70%), as determined after gallbladder cannulation. Heterozygous *Abcg8*<sup>+/-</sup> mice that were not sitosterolaemic showed an intermediate phenotype with respect to biliary cholesterol secretion (-34%). In a separate study, Plösch *et al.*<sup>38</sup> reported that the cholesterol content of gallbladder bile was decreased by ~60% in sitosterolaemic mice in which the *Abcg5* gene alone was disrupted: no heterozygous mice were included in this particular study. It is of interest to note that hepatic expression levels of *Abcg5* and of *Abcg8* were reduced in the mice in which the respective partner gene was selectively disrupted,<sup>37,38</sup> possibly as a consequence of the close proximity of both genes.

All together, these data support an important role of the *Abcg5/Abcg8* heterodimer in control of biliary cholesterol secretion. However, some issues warrant further evaluation to establish its exact role in the secretory process. First, Kusters *et al.*<sup>36</sup> observed that diosgenin-induced hypersecretion of cholesterol into bile does not require induction of *Abcg5/Abcg8* expression. In addition, it should be noted that cholesterol secretion is not completely abrogated in the *Abcg5/Abcg8* double knock out mouse and that there is still a considerable amount (30-40%) of biliary cholesterol secretion left in both the *Abcg5* and *Abcg8* single knock outs.<sup>37,38</sup> Surprisingly, the cholesterol content of gallbladder bile of *Abcg5*-deficient mice was remarkably enhanced by treatment of these animals with a synthetic LXR agonist, to a similar extent as observed in wild-type mice,<sup>38</sup> in spite of the fact that hepatic *Abcg8* mRNA level was not induced. Combined, these data suggests that alternative secretory mechanisms, possibly independent of *Abcg5/Abcg8*, may exist.

## **Mechanisms of intestinal cholesterol absorption**

Intestinal cholesterol absorption has long been considered to represent primarily a passive process, in spite of the fact that it was recognized decades ago that the process is selective in the sense that dietary cholesterol is absorbed relatively efficiently while structurally similar plant sterols and other non-cholesterol sterols are not. After hydrolysis of the small portion of dietary cholesterylester and solubilization by mixed bile salt/(phospho)lipid micelles, cholesterol was supposed to traverse the unstirred waterlayer where the micelles subsequently disintegrated in the local acid microclimate to deliver their cargo at the enterocytic membrane. Size and composition of the bile salt pool<sup>39,40</sup> as well as the amount of phospholipids present

in the intestinal lumen<sup>41,42</sup> were shown to exert regulatory actions on the amount of cholesterol that is ultimately absorbed. The last few years, however, the paradigm of passive enterocytic cholesterol uptake has changed considerably. Cholesterol absorption has been shown to be saturable and to display very large person-to-person variation. Several groups have worked intensely to identify and characterize the proteins involved. Scavenger receptor (SR)-B1 appeared to be a good candidate. This protein was shown to be expressed at the apical membranes of enterocytes of mainly duodenum and jejunum, exactly the sites where most cholesterol is likely to be absorbed.<sup>43</sup> In isolated brush border membrane vesicles cholesterol uptake could be inhibited by the SR-B1 ligand apoA-I and also by antibodies against SR-B1.<sup>44,45</sup> In addition, the recently developed specific inhibitor of cholesterol absorption, ezetimibe, was found to bind to SR-B1.<sup>46</sup> However, SR-B1 null mice absorbed even more cholesterol than the corresponding wild-type mice did, indicating that SR-B1, if indeed involved in transport, is at least redundant. It can not be excluded that the SR-B1 null mice have compensated for their defect by upregulation of other cholesterol transporters, but as far as we know this has not yet been studied in detail.

## Has the “real cholesterol transporter” been identified ?

In a very recent paper,<sup>47</sup> the identity of a prime candidate for the putative cholesterol uptake transporter has been revealed. Altmann and his colleagues searched human and rodent expressed sequence tag (EST) databases for sequences highly expressed in the intestine that contained several characteristic “transporter” features, *i.e.*, transmembrane domains, extracellular signal sequences, sites for N-linked glycosylation, but, in addition, also a sterol-sensing domain. These domains are found in a number of important proteins involved in cholesterol metabolism, including HMGCoA reductase, Niemann-Pick C1 (NPC1) and Sterol Regulatory Element Binding Protein Cleavage-Activating Protein (SCAP). From their analysis only a single credible candidate gene emerged: the rat homologue of *Niemann-Pick C1 Like 1 protein* (NPC1L1). NPC1L1 has ~50% amino acid homology to NPC1.<sup>48</sup> The latter protein functions in intracellular cholesterol trafficking and is defective in the inborn cholesterol storage disease Niemann Pick Type C. In contrast to *NPC1*, which is ubiquitously expressed, *NPC1L1* appeared to be predominantly expressed in the small intestine in humans, rats and mice. Much lower expression levels were observed in liver, gallbladder, testis and stomach. In the rat small intestine, *NPC1L1* mRNA levels varied along the duodenum-ileum axis with peak expression in the proximal jejunum, *i.e.*, the site where most of the cholesterol is thought to be absorbed. NPC1L1 protein levels showed a similar distribution pattern along the length of the small intestine. In the jejunum, *NPC1L1* mRNA was confined to enterocytes and the protein appeared to be predominantly localized apically, *i.e.*, close to or at the plasma membrane facing the intestinal lumen.

NPC1L1-null (*Npc1l1*<sup>-/-</sup>) mice were created to establish the actual role of the protein in cholesterol absorption. NPC1L1-deficiency did not affect development, fertility or any hematological or plasma parameter that was measured. Intestinal morphology was normal. Plasma cholesterol and triglyceride levels were similar in knock out and wild-type littermates while a significantly lower hepatic cholesterylester content was observed in the *Npc1l1*<sup>-/-</sup> mice. Fractional cholesterol absorption rates, determined by a fecal dual isotope method, were  $51 \pm 3\%$  and  $45 \pm 4\%$  in wild-type (*Npc1l1*<sup>+/+</sup>) and heterozygous *Npc1l1*<sup>+/-</sup> mice, respectively, but only  $16 \pm 0.4\%$  in *Npc1l1*<sup>-/-</sup> mice. Addition of cholate to the diet did not improve cholesterol absorption in the latter, indicating that bile salt deficiency is not the cause of cholesterol malabsorption in these animals. Interestingly, ezetimibe treatment reduced cholesterol absorption efficiency in wild-type mice to exactly the value seen in non-treated *Npc1l1*<sup>-/-</sup> mice while the drug had no additional effect in these knock-outs. Together, these data indicate that NPC1L1 plays an essential role in ezetimibe-sensitive cholesterol absorption and that part of the absorption process (~ 30% of total in this particular mouse strain) is NPC1L1-independent. Acute experiments employing radiolabeled cholesterol demonstrated that uptake by the enterocytes was drastically reduced in the *Npc1l1*<sup>-/-</sup> mice, supporting a role of the protein in the uptake of cholesterol across the apical membrane of the enterocytes. These data are of great importance for our understanding of the cholesterol absorption process. Obviously, identification of NPC1L1 as a *bona fide* cholesterol transporter awaits demonstration of actual transport activity in appropriate systems. In addition, a number of other issues remains to be addressed. For instance, it should be demonstrated that ezetimibe really binds to or interacts with the NPC1L1 protein. It was reported that attempts in this direction have been unsuccessful so far.

In this context it is highly interesting that Smart *et al.*<sup>49</sup> very recently identified annexin2 (ANX2) and caveolin1 (CAV1) as potential important components of the intestinal sterol transport machinery that may also be targeted by ezetimibe. Complexes of CAV1 and ANX2 with cyclophilins A and 40, have been implicated in trafficking of exogenous cholesterol from caveolae at the plasma membrane to the endoplasmic reticulum. Studies in zebrafish larvae using morpholino oligonucleotide antisense technology revealed that deletion of ANX2 prevented complex formation as well as processing of a fluorescent cholesterol reporter and results in reduced sterol mass. Exposure of fish embryos to ezetimibe completely disrupted the complex, with CAV1 and ANX2 detected only as monomers. Feeding of ezetimibe to chow-fed C57BL/6 mice did not affect complex stability in enterocytes but, intriguingly, when mice were fed a cholesterol-containing Western-type diet the complex did become sensitive to disruption by ezetimibe. Likewise, ezetimibe treatment disrupted the CAV2-ANX2 complex in hypercholesterolaemic LDL receptor-deficient mice. Furthermore, it was shown by immunoprecipitation on enterocytes followed by mass spectrometry that cholesterol selectively co-precipitated with the complex, which was prevented by pretreatment of the enterocytes with ezetimibe. Experiments in CaCo2 cells revealed that

ezetimibe itself co-precipitated with CAV1 but not with ANX2 or with cyclophilin A. Thus, these data suggest that ezetimibe disrupts the CAV1-ANX2 complex through a direct interaction with CAV1 protein, implying an intracellular site of action of drug. Whether or not interactions between NPC1L1 and CAV1-ANX2 are operational at some stage of the cholesterol absorption process remains elusive for the moment.

## **Controlled efflux to the intestinal lumen as determinant of cholesterol absorption efficacy ?**

In addition to a role of proteins in cholesterol uptake, there is good evidence now that ABC transporter proteins are involved in efflux of sterol from the enterocytes to the intestinal lumen and that the efficiency of the absorption process is, at least in part, governed by this “reflux” system. The function of ABCG5/G8 in biliary cholesterol secretion has been discussed above. Both proteins are also expressed in the intestine, mainly in jejunal sections, where they are responsible for the efflux of plant sterols as indicated by the greatly increased absorption of plant sterols in patients with sitosterolemia.<sup>21,22</sup> Since these proteins mediate cholesterol secretion from liver to bile one may assume that they exert a similar activity in the intestine. Indeed, overexpression of both human proteins reduced cholesterol absorption efficiency and greatly increased fecal neutral sterol loss.<sup>32</sup> The role of the intestine in cholesterol homeostasis has for long been confined to its absorptive function towards bile- and diet-derived cholesterol. However, the notion that the intestine itself may function as an important secretory organ for cholesterol is novel. Plösch *et al.*<sup>30</sup> studied the effect of dietary administration of the LXR agonist T0901317 in C57Bl6 and in DBA/1 mice. By determination of biliary cholesterol secretion and fecal neutral sterol loss, the net intestinal transport could be estimated. In C57Bl/6 mice, the intestine secreted more cholesterol than was (re)absorbed and this net secretion tripled during treatment with T0901317. A similar conclusion can be drawn from the work of Yu *et al.*<sup>32,33,50</sup> In the *Abcg5/g8* double knock-out mice, biliary cholesterol secretion is almost absent, yet their endogenous neutral sterol excretion is barely affected. Conversely in the *ABCG5/G8* overexpressor neutral sterol output was strongly increased. In these mice fecal neutral sterol output was more than fivefold increased to reach a value of about 110  $\mu\text{mol/day/100g BW}$ . Unfortunately, biliary output was not quantified making a direct comparison with the data of Plösch *et al.*<sup>30</sup> impossible. The increase in fecal neutral sterol excretion of about 90  $\mu\text{mol/day/100g BW}$  is equivalent to 60  $\text{nmol/min/100g BW}$  of biliary cholesterol flow on the assumption that no biliary cholesterol is reabsorbed. This underestimated value is about 3fold higher than the (already very high) total T0901317-induced biliary cholesterol secretion reported by Plösch *et al.*<sup>30</sup> Accordingly, one has to conclude that also in the experiments of Yu *et al.*<sup>33</sup> substantial net cholesterol secretion from the intestine occurs.

The origin of this net intestinal cholesterol secretion is an intriguing issue. It is generally assumed that there is no appreciable cholesterol flux from the circulation to the enterocyte. When this assumption would hold in the now rapidly changing understanding of cholesterol fluxes, the cholesterol secreted into the intestine can only be derived directly from the enterocytes. In the experiments from Plösch *et al.*<sup>30</sup> intestinal HMG-CoA reductase expression did not change. So, either the enzyme in the intestine is regulated post transcriptionally or the extra cholesterol is not derived from the *de novo* synthesis. Yu *et al.*<sup>50</sup> did not find any effect of the LXR agonist on intestinal neutral sterol output in the Abcg5/g8 double knock-out mouse indicating that the heterodimer is fully responsible for the LXR-mediated effect on neutral sterol excretion. There have been reports for a role of other genes in regulation of sterol uptake.<sup>51,52</sup> Whether these genes are involved in cholesterol uptake or in additional efflux pathways is not clear at the moment and requires further investigation.

## **Intestinal lipoprotein formation as part of the cholesterol absorption cascade**

Cholesterol that has entered the enterocyte traffics to the endoplasmic reticulum to be esterified by acylCoA:cholesterol acyltransferase-2 (ACAT2), the ACAT isoform that is highly expressed in intestine and liver. The mechanisms by which cholesterol and plant sterols move to the endoplasmic reticulum are largely unknown but, as outlined above, several chaperones of vesicular transport have been implicated in the process. Studies in ACAT2-deficient mice<sup>53</sup> revealed that the fractional absorption of dietary cholesterol was not affected as compared to wild-type controls when the animals were fed a low-cholesterol chow diet. Yet, when animals were fed a high-fat, high-cholesterol diet, fractional cholesterol absorption was much less in ACAT2-deficient mice than in controls. As a consequence, these animals were protected from diet-induced hypercholesterolaemia and gallstone formation. Thus, ACAT2 seems to be of regulatory importance in cholesterol absorption in mice only when cholesterol intake is at an appreciable level, as also appeared to be the case for ABCG5/ABCG8.<sup>32,33</sup> It has been postulated that selectivity of intestinal sterol absorption is, at least in part, related to sterol selectivity of ACAT2: the enzyme shows a strong preference for cholesterol rather than sitosterol.<sup>54</sup> In this scenario, microsomal sterols not esterified by the actions of ACAT2 would be transported back to the apical plasma membrane to be effluxed by the ABCG5/ABCG8 heterodimer. The nature of this apical transport process is still unresolved. Non-selective ACAT inhibitors, exemplified by the sulfamic acid phenyl ester avasimibe, have been developed and were shown to reduce the absorption of dietary cholesterol, to impair the secretion of VLDL particles by liver cells and to reduce the extent of atherosclerosis in animal models (*e.g.*, Delsing *et al.*<sup>55</sup>). Avasimibe is currently in clinical trials and has, for instance,

been shown to induce a modest reduction of triglycerides and VLDL-cholesterol with no significant changes in LDL-cholesterol in subjects with combined hyperlipidemia.<sup>56</sup> Avasimibe monotherapy was not effective in subjects with homozygous familial hypercholesterolemia,<sup>57</sup> and showed only a modest synergistic effect on total cholesterol levels when given with atorvastatin. Thus, the clinical benefit of this particular drug appears limited for the moment: it may be that increasing selectivity of novel drugs towards ACAT2, perhaps with an intestine-specific profile, will improve effectivity of this approach.

A final crucial event in the cholesterol absorption process involves the incorporation of newly esterified cholesterol molecules, together with a small amount of unesterified sterol, triglycerides and phospholipids, along with apolipoprotein B48 into nascent chylomicrons that are delivered into the lymphatics. The availability of apoB48 is essential for cholesterol absorption: mice lacking functional apoB48 in their intestine do not absorb measurable quantities of cholesterol.<sup>58</sup> The assembly of chylomicrons, like that of VLDL in hepatocytes, is facilitated by the microsomal triglyceride transfer protein (MTP). The remarkable effects of MTP inhibitors on plasma lipid concentrations may involve consequences of reduced cholesterol absorption but there safety concerns with respect to the use of these drugs that need to be solved.<sup>59</sup>

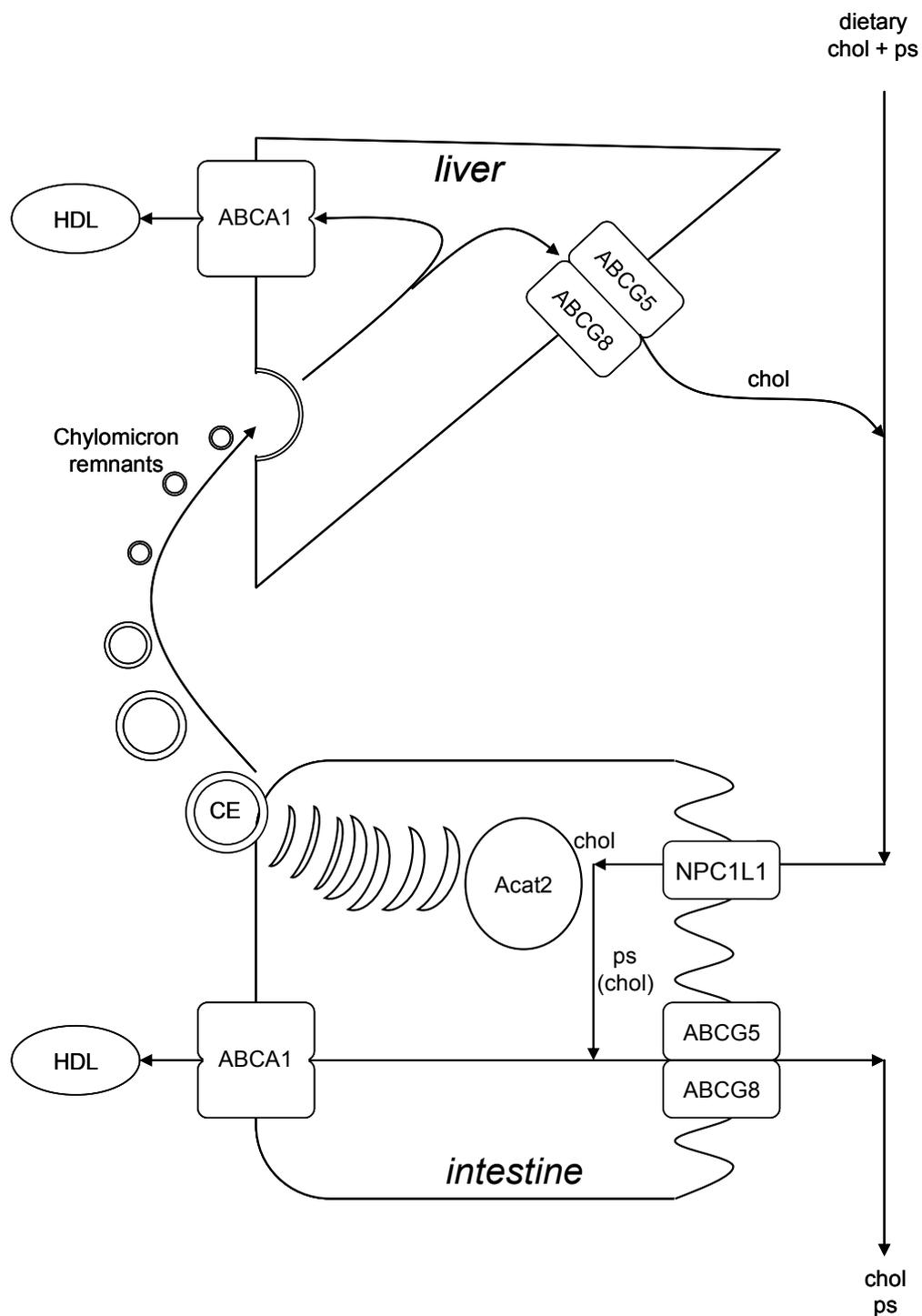
It has been known for decades that the intestine is an important source of HDL but whether or not intestinal HDL serves specific physiological functions, for instance in cholesterol absorption, is not clear. ABCA1, crucial for HDL formation, is highly expressed in enterocytes of the small intestine and present at the basolateral plasma membrane.<sup>60</sup> In a chicken model of ABCA1 dysfunction, Mulligan *et al.*<sup>61</sup> showed that the percentage of orally administered <sup>14</sup>C cholesterol appearing in plasma was reduced by 79% and that radiolabeled cholesterol accumulated in the intestinal wall. From these data, the authors concluded that ABCA1 regulates the efflux of cholesterol from the basolateral membrane during absorption of dietary cholesterol in chicken which, unlike mammals, lack lymphatic contribution to intestinal lipid absorption. The quantitative importance of this pathway in overall absorption in mammals remains, therefore, to be established. Measurement of fractional cholesterol absorption in ABCA1-deficient mice by different dual isotope measurements revealed no marked differences in comparison to wild-type controls when animals were kept on low cholesterol diets.<sup>62,63</sup> Since biliary cholesterol content is not affected in ABCA1-deficient mice,<sup>64</sup> it is likely that absolute amounts of cholesterol absorbed were also not strongly affected in the absence of ABCA1 under these experimental conditions. Likewise, measurement of fractional cholesterol absorption in a single patient with Tangier disease revealed a value in the “normal range”.<sup>65</sup> Interestingly, fractional cholesterol absorption was significantly higher in *ABCA1*<sup>-/-</sup> mice than in wild-type mice when fed a high-cholesterol Western type diet.<sup>62</sup> Evidently, the mechanisms of action of and the interactions between the various pathways involved in cholesterol absorption, that may be different under various dietary conditions, need further exploration.

## Concluding remarks

Since the beginning of this century insight in mechanisms involved in regulation of cholesterol handling in liver and intestine has increased considerably, as summarized in the figure (Figure 1). Separate proteins active in cholesterol import and efflux have been characterized in the intestine, constituting a so-called substrate cycle. It has been known for many years that in humans the responses to dietary cholesterol and cholesterol-lowering medication as well as several parameters of cholesterol metabolism (fractional absorption rate, conversion to bile salts, biliary secretion rates) show wide interindividual variations. Subtle difference in the activity of one or both of the arms of the substrate cycle may account for these variations. Although most of the regulatory modulators active *in vivo* have not yet been elucidated the available knowledge allows development of drugs specifically targeted to these transport systems. Ezetimibe and dietary plant sterols probably already fulfill these criteria. In this chapter we have shown that biliary secretion may not be the only pathway via which cholesterol can be excreted from the body. A direct route has to exist as well although it is not yet clear which carriers and transcellular mechanism account for this activity. Nevertheless elucidation of the steps involved may provide attractive additional targets to stimulate cholesterol disposal from the body. Such strategies potentially would be powerful complements to the traditional therapies aimed at decreasing cholesterol synthesis and together with statins may lead to potent regression of atherosclerotic lesions.

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**Figure 1:** Schematic representation of cholesterol transport in liver and intestine and the proteins involved (chol: cholesterol; ps: plant sterol).

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