The ABC of cholesterol transport

Plösch, Torsten

CHAPTER 1

General introduction

Aim of the thesis

Introduction

Sterols fulfill several indispensable roles in all eukaryotic cells. Cholesterol is the most important sterol in mammals. As described below, it is an integral part of plasma and organelle membranes as well as a precursor of important molecules like bile salts and steroid hormones. During embryogenesis, cholesterol is involved in pattern formation in the developing animal. Defects in cholesterol synthesis or intracellular routing have devastating consequences for the individual. In humans, the Smith-Lemli-Opitz syndrome, desmosterolosis and Niemann-Pick CI disease are examples for inherited diseases caused by mutations of genes involved in cholesterol metabolism. However, cholesterol and sterols that are ubiquitously present in the diet also pose a potential danger. These sterols are critically involved in the development of atherosclerosis which is a major health risk in Western societies. Hence, cellular cholesterol homeostasis and plasma cholesterol levels have to be regulated very strictly. This regulation is discussed in detail below.

Cholesterol is an isoprenoid molecule with 4 hydrocarbon rings and a hydrocarbon side chain at C17 (see Figure 1). It contains 27 carbon atoms and possesses a hydroxylgroup at C3. Cholesterol is a very hydrophobic molecule, with only limited polarity due to the hydroxylgroup. Its hydrophobicity is on the one hand responsible for its beneficial property to control cell membrane fluidity; it on the other hand makes it very difficult to handle in the aqueous environment of the body, both within cells and between cells. Therefore, sophisticated mechanisms exist to transport cholesterol to its various destinations.

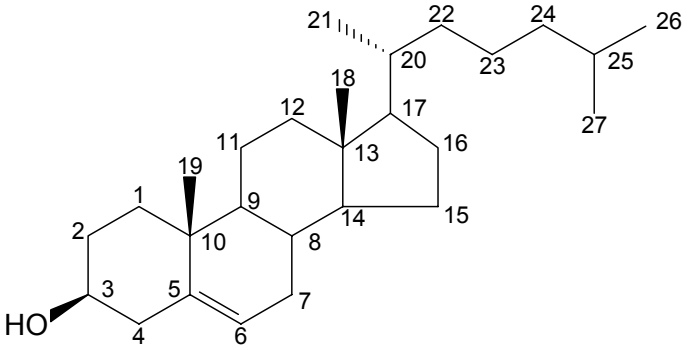


Figure 1: Structure of cholesterol with the standard carbon numbering according to IUPAC recommendations.

Cholesterol metabolism

Cholesterol is synthesized from acetyl-CoA units via the mevalonate pathway. Synthesis starts with the formation of 3-hydroxy-3-methylglutaryl CoA (HMG CoA) from acetyl CoA and acetoacetyl CoA. HMG CoA is subsequently reduced to mevalonate. This rate-controlling, irreversible step of cholesterol synthesis is regulated by the enzyme HMG CoA reductase.[1] The mRNA expression levels of this enzyme are used as surrogate marker for cholesterol formation throughout this thesis, although it is realized that this parameter does not directly reflect the actual cholesterol synthesis rate.

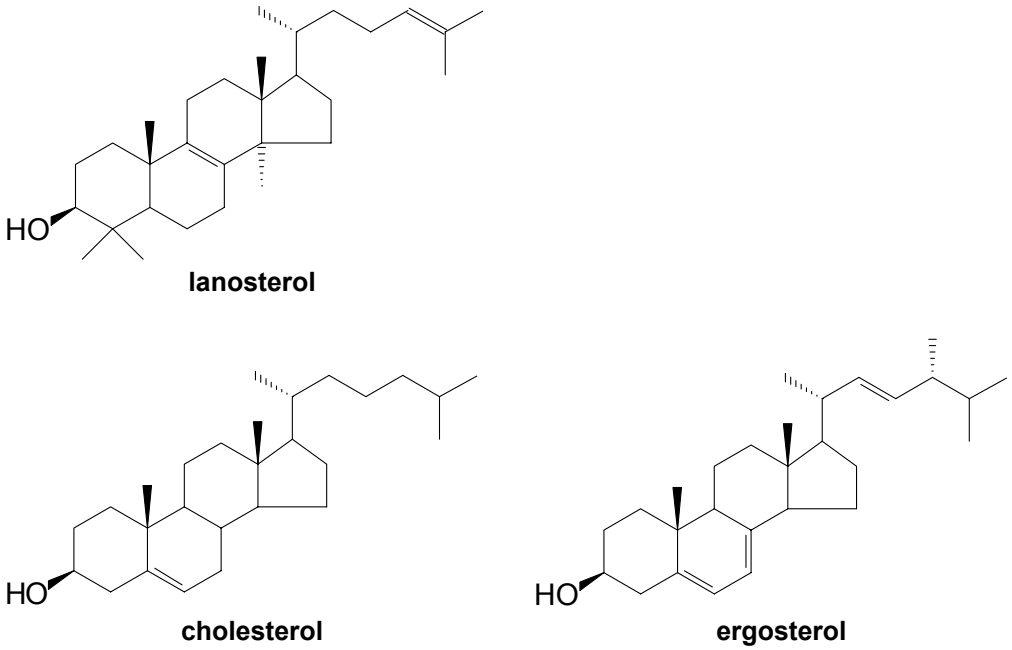


Figure 2: Lanosterol is the precursor of both cholesterol in mammals and ergosterol in fungi.

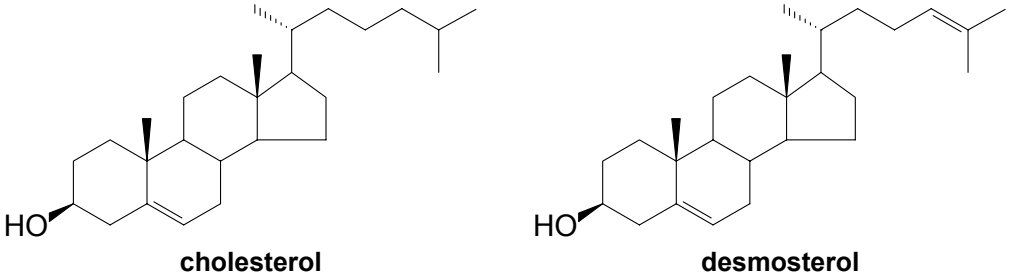
The following synthesis steps include, amongst others, conversion of mevalonate to isopentenyl pyrophosphate, condensation to squalene, and finally cyclization of squalene to form lanosterol. Lanosterol is the precursor of cholesterol as well as of ergosterol, the main sterol of fungi (Figure 2).

Cholesterol is then incorporated into cell membranes, stored as cholesteryl ester or used as substrate for downstream reactions. The largest part of the cholesterol used for downstream reactions is eventually transformed into bile salts (see below). A quantitatively less important part is used for the synthesis of steroid hormones.

Functions of cholesterol in mammals

Most of the cholesterol present in the body resides in cell membranes. The molecule is situated in parallel to the fatty acid chains of the phospholipids, with the polar hydroxyl group close to phospholipid head group. Membrane fluidity is largely dependent on the concentration of sterols present: due to their rigid structure, sterols reduce the fluidity of the membrane. This, in turn, determines membrane properties as permeability and stability. A well-known example is the insulating myelin shield of neurons with it high cholesterol content.[2]

The relatively mild phenotype of 24-dehydrocholesterol reductase knockout mice (*Dhcr24*$^{-/-}$),[3] which do not synthesize cholesterol but desmosterol (Figure 3), may indicate that other sterols are, in principle, able to fulfill the role of cholesterol in membranes. In the human equivalent, desmosterolosis,[4] however, pattern formation in embryogenesis is impaired, probably caused by absence of cholesterol during critical phases of development.



cholesterol **desmosterol**

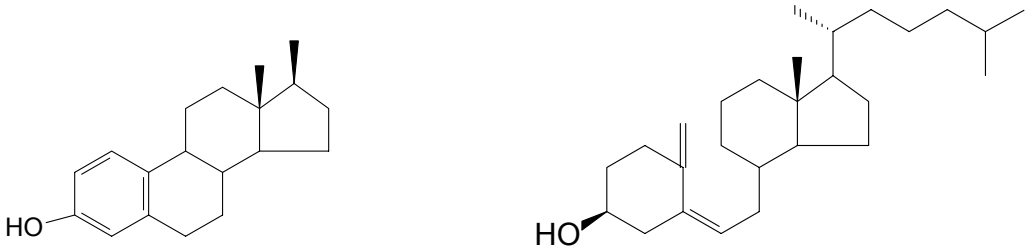
Figure 3: Desmosterol is the main sterol in 24-dehydrocholesterol reductase knockout mice.

Cholesterol is covalently linked to hedgehog proteins which are crucial for pattern formation in animals from *Drosophila* to human.[5] In addition, cholesterol is essential for the function of the myelin sheaths and therefore indispensable for brain development.[2] In mice, delivery of cholesterol from the mother to the embryo may compensate for the deficiency during embryogenesis.[6,7]

A second important function of cholesterol is that as a precursor of bile salts (see page 14, Figure 5) and steroid hormones (Figure 4). Bile salts synthesis takes place exclusively in the liver (see below).[8] In contrast to cholesterol, bile salts are at least partially polar. The major function of bile salts is to act as physiological detergents: they emulsify droplets of dietary lipids and lipophilic vitamins, making them available for absorption in the intestine. Hence, absence of bile salt secretion leads to malabsorption of lipophilic vitamins and fatty acids.

Steroid hormones (*i.e.*, progestagens, glucocorticoids, mineralocorticoids, androgens, and estrogens; Figure 4) are potent hormones which are involved in the regulation of a variety of processes, *e.g.*, reproduction and metabolism. The receptors for steroid hormones, and especially the estrogen receptor, are the best characterized of all nuclear receptors, which will be discussed below.

Lastly, the immediate cholesterol precursor 7-dehydrocholesterol can be photolyzed by UV light to yield previtamin D3. Hence, also the actions of vitamin D (Figure 4) ultimately depend on the cholesterol synthesis pathway. The very early isoprenoid intermediates of the cholesterol synthesis pathway are precursors for a variety of structurally extremely different molecules involved in many different metabolic pathways. Therefore, defects in the early steps are incompatible with life.



estradiol-17ß **vitamin D (cholecalciferol)**

Figure 4: Structures of the steroid hormone estradiol-17ß, an estrogen, and of vitamin D, which is derived from 7-dehydrocholesterol.

Harmful effects of cholesterol: atherosclerosis

The hydrophobicity of cholesterol, which makes it difficult to handle in an aqueous environment, is associated with the main problems associated with increased plasma cholesterol concentrations, *i.e.*, atherosclerosis. Atherosclerosis is a prerequisite for the majority of cases of coronary heart disease, which is the most prevalent cause of death in industrial countries.[9]

The development of atherosclerosis is a process starting already early in life, possible already *in utero*.[10] Lipids such as cholesterol, derived from lipoproteins, accumulate in macrophages in the vessel wall.[11] Cholesterol-loaded macrophages, so-called *foam cells* because of their microscopically visible, opalescent lipid content, form *fatty streaks* in the endothelium of the vessel. Due to the interaction with inflammatory blood cells, an atherosclerotic plaque develops which subsequently can block the vessel or rupture and subsequently block capillaries at other places, *e.g.*, the heart or the brain.[9]

There is convincing epidemiological data available demonstrating the correlation between plasma cholesterol levels in the various lipoprotein fractions and coronary heart disease (*e.g.*, refs. 12-16). In short, high levels of HDL cholesterol ("good" cholesterol) have been correlated with a protective effect, high levels of LDL cholesterol ("bad cholesterol") with a deteriorating effect. It was generally believed that the direction of cholesterol transport in the body is the underlying mechanism for these correlations: LDL is a vehicle for cholesterol supply to peripheral cells (and, therefore, to macrophages), whereas HDL is important for reverse cholesterol transport from the periphery to excretory organs, *i.e.*, the liver. This view is currently under discussion, as several papers have indicated that plasma HDL content is not rate-controlling for reverse cholesterol transport (*e.g.*, refs. 17-19). Hence, several alternative explanations for the lipoprotein-related effects have been proposed (see Sviridov and Nestel[20] for review).

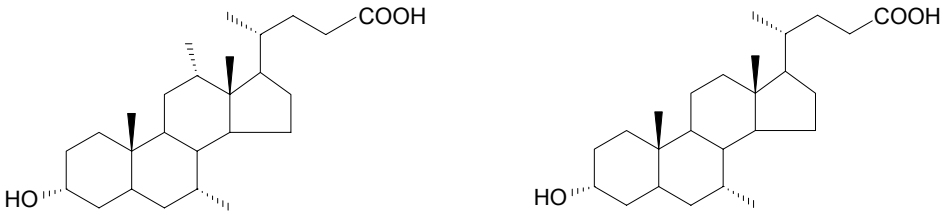
Bile salt synthesis

Conversion into bile salts comprises the final destination for most of the cholesterol in the body. In humans, about 500 mg cholesterol is converted into bile salts per day. Bile salt synthesis takes exclusively place in the liver. It involves the action of 17 different enzymes and can follow two major pathways, named the "classic" or neutral pathway and the "alternative" or acidic pathway. The classic pathway accounts for the majority of bile salt synthesis, *i.e.*, for approximately 75 % in mice and for 90 % in humans (see ref. 8 for review). However, it may be even more significant under certain circumstances.[21]

Characteristic for the classic pathway is the conversion of cholesterol to 7α-hydroxy-cholesterol by the enzyme cholesterol-7α-hydroxylase (Cyp7a1).[22] As this step determines the flux through the classic route, the regulation of *Cyp7a1* expression is of crucial importance. *Cyp7a1* mRNA levels are therefore indicative for bile salt synthesis via the classic pathway. End products of the classic pathway are cholate and chenodeoxycholate (human; see Figure 5) or cholate and muricholate in mice. Cholate synthesis involves a reaction mediated by 12α-hydroxylase (Cyp8b1); the ratio between cholate and either chenodeoxycholate or muricholate is consequently determined by the activity of Cyp8b1 present in the liver.

First step of the alternative pathway is the formation of 27-hydroxycholesterol by the enzyme sterol 27-hydroxylase (Cyp27).[22] By-products of this step are other oxysterols, namely 24- and 25-hydroxycholesterol, which can also be used for bile salt synthesis.[22] Ultimately, chenodeoxycholate is the main end product of this pathway.

Typically, bile salts are conjugated with either taurine or glycine before they are excreted to bile.[23] During the enterohepatic circulation (see below), intestinal bacteria modify bile salt structures which yields secondary bile salts, *e.g.*, lithocholate and deoxycholate.[24] As about 95 % of the bile salts re-enter the enterohepatic circulation, the bile salt pool of the body consists of a mixture of primary and secondary bile salts.



cholate **chenodeoxycholate**

Figure 5: Structures of the major mammalian primary bile salt species cholate and chenodeoxy-cholate. Muricholate replaces chenodeoxycholate in mice. Please note that they are readily conjugated with glycine or taurin.

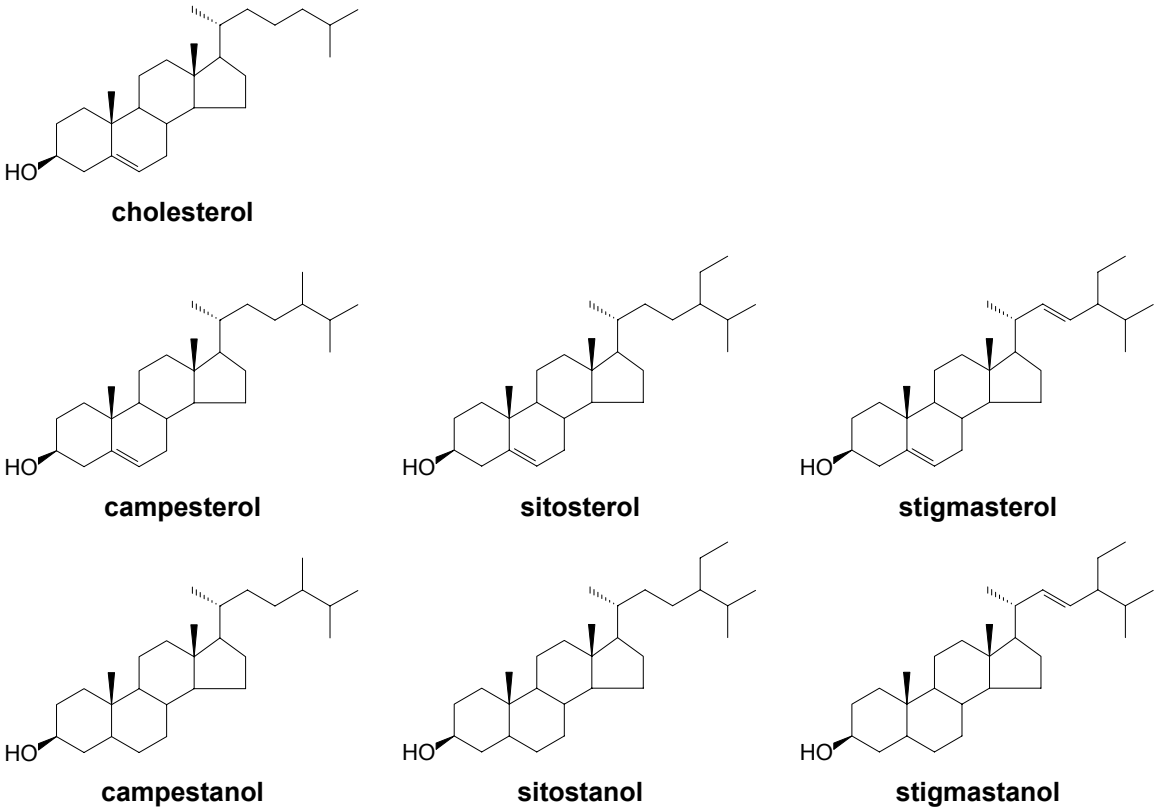


Figure 6: Structures of the major plant sterols and stanols compared to cholesterol. Plant sterols differ from cholesterol by the structure of the side chain, namely the group attached at C24 and the number of double bonds. Stanols, in contrary to sterols, do not possess a C5-C6 double bond.

Plant sterols

Plants produce a variety of different sterols. In parallel to the functions of other sterols in animals, plant sterols are membrane constituents[25] as well as precursors for plant hormones and other secondary metabolites, *i.e.*, substances interfering with pathogens and insects.[26-28] Sterol composition differs between species as well as between tissues and is furthermore influenced by developmental stage and environmental conditions. One prominent example is corn (*Zea mays*), in which 61 different sterols and related compound have been identified.[29]

The most prevalent sterols in plants are the 24-ethylsterols ß-sitosterol and stigmasterol and the 24-methylsterols campesterol and 22-dihydrobrassicasterol.[28] As demonstrated in Figure 6, these sterols differ only in the structure of their side chains. Cholesterol is also present in plants, usually in low concentrations. However, in some plant families and during some developmental stages, cholesterol can account for up to 70 % of total sterols.[30,31] Most of the sterols are found with a free 3ß-hydroxyl group, but esterification with long-chain fatty acids or phenolic acids does occur.[28] In addition to sterols, the corresponding stanols are present in plants, although in lower concentrations. Plant stanols differ from the corresponding sterols by a double bond between C5 and C6 (Figure 6).

On a Western-type diet, the dietary intake of plant sterols is in the same order of magnitude as that of cholesterol. In The Netherlands, for example, the median daily intake was 160, 26, 14, and 98 mg for cholesterol, campesterol, stigmasterol, and ß-sitosterol, respectively.[32]

ATP-binding-cassette (ABC) transporters

The plasma membrane forms a barrier between the cytosol and the extracellular space. Therefore, mechanisms must exist to transport substances from one compartment to another, *i.e.*, into the cell or out of the cell. In addition, transport systems are necessary to enable exchange between the cytosol and the cell organelles. ATP-binding-cassette (ABC) transporters are a large family of proteins which mediate transport of a wide variety of substrates across different cellular membranes (out of bacterial and eukaryotic cells), processes driven by the hydrolysis of ATP.[33,34]

Most mammalian ABC transporters contain two ATP-binding and two transmembrane domains.[33,35] In the ATP-binding domain the highly conserved Walker A and Walker B motifs are present which are involved in ATP binding and hydrolysis. The transmembrane domain is formed by 6 membrane-spanning α-helices. Some ABC-transporters contain only one ATP-binding site and one set of 6 transmembrane helices. They are therefore called "half-transporters" and are considered to act as homodimers or as heterodimers with other half-transporters.

In the human genome, 48 ABC transporters have been identified up to now.[33,36,37] They are classified in 7 groups (A-G) based on their structure and homology. In the following sections, the transporters of particular relevance for the studies described in this thesis will be discussed.

The ABCA-family

Out of the 12 members of the ABCA family, Abca1 has been studied in most detail (see Chimini *et al.*[38] for review). It is expressed in a variety of different tissues, with high expression levels in hepatocytes, enterocytes and macrophages.[39-41] Abca1 facilitates the efflux of phospholipids and cholesterol from cells to lipid poor, nascent HDL particles (pre-ß-HDL) and is essential for the formation of HDL. It has been proposed that Abca1 primarily binds to and promotes phospholipid efflux to ApoA-I, secondarily followed by cholesterol.[42] Abca1 is localized at the basolateral membrane of polarized cells such as hepatocytes and enterocytes, with substantial amounts of the protein continuously *en route* between the cell surface and late endocytic vesicles.[43,44] Recent data indicate that it may exert its function in the late endosomal compartment.[45] Expression of Abca1 is regulated by nuclear receptors, particularly by LXR/RXR.[46-48]

Confirming its role in HDL formation, absence of functional ABCA1 protein due to mutations in the *ABCA1* gene causes Tangier disease which is characterized by absence of HDL and accumulation of cholesteryl esters in various tissues.[49-51] Interestingly, a spontaneously mutated *Abca1* gene has been identified in chicken.[52] In this thesis, a mouse model of Abca1-deficiency was used to study the influence of HDL in reverse cholesterol transport (Chapter 2).[19] Besides its role in HDL formation, Abca1 has functions in inflammatory processes, apoptosis[38] and influences male fertility.[53]

The ABCB-family

Of the ABCB family, two members are of outstanding interest for the work described in this thesis, namely Mdr2/MDR3 (Abcb4) and Bsep (Abcb11). Both are almost exclusively expressed in the liver.

Mdr2/Abcb4 P-glycoprotein (called MDR3/ABCB4 in humans) is present at the canalicular membrane of hepatocytes, where it is critically involved in secretion of phosphatidylcholine across the membrane into bile. The function of Mdr2 has been extensively studied in mice lacking a functional *Mdr2* gene (*Mdr2*$^{-/-}$ mice).[54] *Mdr2*$^{-/-}$ mice do not excrete phospholipid into bile, but bile salt secretion is normal.[55] Biliary phospholipids protect the canalicular membrane against the deterging action of bile salts. Consequently, *Mdr2*$^{-/-}$ mice show severely damaged, inflamed bile ducts with portal fibrosis.[54] As hepatobiliary cholesterol and phospholipid secretion are functionally coupled,[56] cholesterol secretion is strongly impaired in mice lacking Mdr2. However, infusion with the hydrophobic bile salt taurodeoxycholate (TDCA) restores hepatobiliary cholesterol secretion without affecting that of phospholipids.[57] As a human counterpart to the *Mdr2*$^{-/-}$ mouse model, MDR3 deficiency has been found to lead to *progressive familiar intrahepatic cholestasis* (PFIC) type 3, a disease characterized by extensive liver damage and increased serum gamma-glutamyltransferase activity. Patients with PFIC3 present with a liver histology very similar to that of the *Mdr2*$^{-/-}$ mice.[58,59]

The bile salt export pump Bsep (also known as Abcb11 or Spgp) transports bile salts across the canalicular membrane.[60,61] Bile salt secretion is the major driving force for bile formation. Therefore, absence of functionally active BSEP causes cholestasis (*progressive familiar intrahepatic cholestasis* (PFIC), type 2) in humans.[62] Bsep expression is regulated via FXR/RXR, with chenodeoxycholic acid as the most effective agonist *in vitro*.[63,64] In this way, rising concentrations of bile salts in the hepatocyte lead to increased Bsep expression which protects against accumulation of toxic bile salts inside the cell (discussed below).[65]

The ABCG-family

Members of the ABCG family are half-transporters which supposedly have to form dimers to become functionally active. Five human members of this family have been characterized so far, but 15 are known from *Drosophila*.[36] In *Drosophila*, these half-transporters can form a

variety of heterodimers with each other, each with a different substrate specificity.[66,67] In mammals, it is currently believed (although not proven) that Abcg5 and Abcg8 form a heterodimer, whereas the remaining ones form homodimers.

Abcg2 (or Bcrp, breast cancer resistance protein), is expressed in the intestine, liver, placenta, and in epithelial cells[68] and in a variety of stem cells.[69] It has been shown to transport chlorophyll-catabolites,[70] metabolites of heme synthesis pathway[71] as well as amphipatic drugs.[72] Its physiological function appears to be protection against accumulation of toxic hydrophobic compounds, for example porphyrin derivatives from the diet (chlorophyll-catabolites) or by-products of heme synthesis.[73]

The genes for Abcg5 and Abcg8 are situated in a head-to-head configuration close to each other, in humans on chromosome 2p21 and separated by 384 base pairs.[74] They are highly expressed in the intestine and the liver. Mutations in either one of them cause sitosterolemia, an inborn error of metabolism characterized by accumulation of plant sterols in the body.[75-77] A naturally occurring mutation in Abcg5, associated with increase plant sterol absorption, has also been described in rats (*Wistar Kyoto inbred*, *spontaneously hypertensive rat*, and *stroke-prone spontaneously hypertensive rat*).[78] Therefore, Abcg5/Abcg8 are thought to limit plant sterol absorption in the intestine and facilitate biliary excretion of plant sterols by the hepatocyte.[76,79] Overexpression of Abcg5 and Abcg8 in transgenic mice leads to reduced absorption of cholesterol in the intestine and to an increased delivery of cholesterol into bile, which indicates that also cholesterol is a substrate of the Abcg5/Abcg8 heterodimer.[80] Expression of Abcg5 and Abcg8 is increased upon the activation of LXR[19,81] and possibly also by liver receptor homolog-1 (LRH-1).[82] Mice lacking either Abcg5,[83] Abcg8,[84] or both Abcg5 *and* Abcg8[85] do not show the same phenotype in all physiological aspects, leaving some space for discussion about their mode of action.

Finally, the Abcg1 protein is highly expressed in macrophages and has been hypothesized to be involved in cholesterol efflux.[86] Abcg1 is an LXR target gene.[87] However, no clear physiological role for Abcg1 could be established so far.[88] The same holds true for Abcg4, the closest relative of Abcg1, which is highly expressed in the brain and the eye.[89] In a very recent paper, elegant *in vitro* studies were performed in which cells were transiently transfected with cDNAs encoding all known murine Abcg-half-transporters and combinations hereof.[90] Abca1 was used for comparison. It could be demonstrated that cells expressing Abcg1 or Abcg4 were able to transfer cholesterol and phospholipids to mature HDL-2 and HDL-3, but not to ApoA-1. In contrast, cells transfected with Abca1 transferred cholesterol to ApoA-1, but not to mature HDL. This is a strong indication that in macrophages Abca1 is responsible for lipidation of lipid-poor pre-ß-HDL, whereas Abcg1 is involved in the lipid-transfer to mature HDL. Abcg4 is hardly expressed in macrophages, but it may have an analogous function in the brain.[89] Nonetheless, additional (*in vivo*) studies are required to confirm these results.

Molecular regulation of cholesterol metabolism by nuclear receptors

The prototype of a nuclear receptor, the estrogen receptor, has been known for a long time in physiology and has been cloned almost 20 years ago.[91] Based on homology of nucleic acid sequences, many other putative nuclear receptors were subsequently discovered without information about their physiological ligands and, hence, their functions. Therefore, these were named *orphan* nuclear receptors. During the last decade, physiological ligands have been discovered for many of these orphans; many of them were found to be or be derived from diet constituents, particularly members of the various lipid classes.[92]

At present, 49 nuclear receptors have been identified in the human genome (see Francis *et al*. for review.[93] They are characterized by a central, highly conserved *DNA-binding domain* (DBD) which specifically targets the corresponding response element (RE), a short recognition sequence of DNA in promoters of genes. The C-terminus of the nuclear receptor is responsible for ligand-binding and for dimerization (*ligand-binding domain*, LBD), whereas the ligand-independent *activation function 1* (AF-1) domain at the N-terminus coordinates the action of co-repressors and co-activators. Nomenclature of nuclear receptors follows an internationally accepted system.[94] A schematic overview of the classes of nuclear receptors is presented in Figure 7.

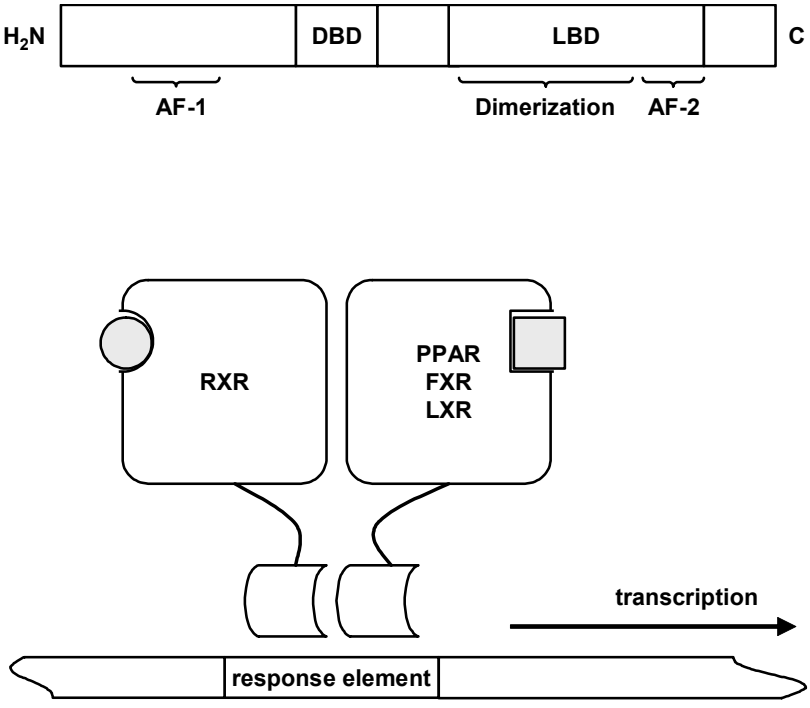


Figure 7: Schematic structure of a nuclear receptor.

Above: Organization of a nuclear receptor.
AF, activation function
DBD, DNA-binding domain
LBD, lipid-binding domain

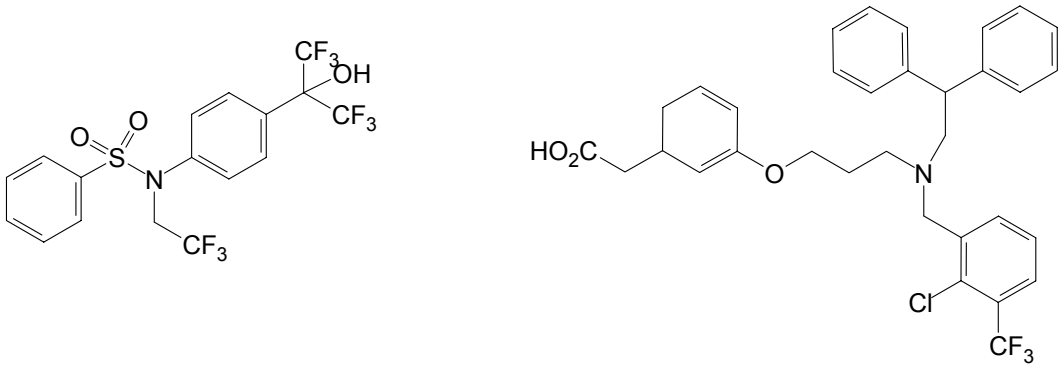
Below: heterodimerization and DNA-binding of nuclear receptors.

Inactivated nuclear receptors generally are bound to co-repressors.[93] Upon ligand-binding, they undergo a conformational change which leads to dissociation of the co-repressor, association of co-activators, and often dimerization with a partner, binding to a promoter site, ultimately resulting in transcription of the target gene. Most nuclear receptors are active as homodimers or heterodimers, a minority acts as monomer. Steroid hormones receptors, for example, form homodimers. Conversely, most of the receptors discussed below heterodimerize with the retinoid X-receptor RXR.[95,96]

The liver-X-receptor LXR

The major player in regulation of cholesterol homeostasis is the liver-X-receptor LXR. Two LXR genes are known, *i.e.*, LXRα (NR1H3), which is highly expressed in the liver, and LXRß (NR1H2) with a more ubiquitous distribution.[97-100] From these two, LXRα has been studied in most detail. Physiological ligands of LXR are oxysterols, oxidized cholesterol derivatives, 24(S),25-epoxycholesterol being the most potent one, and 6α-hydroxy bile salts.[98,101] Oxysterols are formed as by-products during cholesterol synthesis as well as by oxidation of dietary cholesterol.

The LXR system provides a means for cells to "measure" intracellular cholesterol concentrations. If LXR gets activated, the LXR/RXR heterodimer induces transcription of genes involved in cholesterol disposal from cells. Well-known target genes of LXR are *Cyp7a1*[102] (leading to increased catabolism of cholesterol to bile acids), *Srebp-1c*[103] (resulting in increased lipogenesis) and the ABC-transporters *Abca1*, *Abcg1*, *Abcg5* and *Abcg8* (leading to increased removal out of the cell).[47,81,87,104] Activation of LXR by synthetic ligands also leads to increased lipogenesis and to production of large, triglyceride-rich Very-Low-Density-Lipoprotein particles.[105,106] Research described in this thesis makes use of two synthetic, non-steroidal LXR agonists which are highly effective in activating LXR: T0901317 and GW3965 (Figure 8).[48,107]



T0901317 **GW3965**

Figure 8: Structures of the synthetic LXR agonists T0901317 and GW3965.

The farnesoid-X-receptor FXR

The farnesoid-X-receptor FXR (NR1H4) forms heterodimers with RXR upon its binding to bile salts, thereby sensing the level of bile salts inside the cell. FXR is expressed at high levels in the liver and intestine, organs that are normally exposed to bile salts, as well as in the kidney and adrenal cortex.[108] In mice, bile salt synthesis is repressed upon binding of cholate to FXR.[109] Upon activation, FXR reduces the expression of *Cyp7a1* and *Cyp8b1,* therefore limiting bile salt synthesis[110,111] and increases the expression of the bile salt exporter Bsep in hepatocytes.[63] Moreover, it enhances the expression of the short heterodimer partner (Shp)[110] which in turn represses the expression of the Na/taurocholate co-transporting polypeptide (Ntcp). Ntcp is the transport protein responsible for uptake of bile salts from the blood compartment.[65] Taken together, FXR thereby protects the hepatocyte against the toxic effects of bile salts by reducing their synthesis and uptake and increasing their excretion. Interestingly, the active compound of a traditional Indian medicine, guggulsterone, which is used to lower serum LDL and triglyceride levels, has been shown to act as an FXR antagonist.[112] It is suggested that, by inhibition of the suppressive effect of FXR on bile salt synthesis, guggulsterone stimulates the conversion of cholesterol to bile salts, which results in the cholesterol-lowering effects.

The peroxisome proliferator-activated receptors PPAR

Three mammalian peroxisome proliferator-activated receptors are known: PPARα (NR1C1), PPARγ (NR1C2) and PPARδ (NR1C3, also named PPARß). They are all activated by polyunsaturated fatty acids, eicosanoids, and a variety of synthetic ligands (see Willson *et al.*[113] for review). Activated PPARs do heterodimerize with RXR prior to binding to promoter sites in target genes.

PPARα is mainly expressed in the liver, followed by heart and muscle. It is responsible for the major metabolic adaptation to fasting. During fasting, free fatty acids, released from adipose tissue, activate PPARα and thereby initiate transcription of genes responsible for hepatic fatty acid oxidation. Consequently, the liver starts to metabolize fatty acids and to produce keton bodies, providing energy for the periphery.[114]Well-characterized, synthetic ligands for PPARα are the fibrates: their lipid-lowering effects are based on the PPARα-mediated metabolic shift towards fatty acid utilization.[115]

The ubiquitously expressed PPARδ (PPARß) has several functions; besides its role in lipid metabolism, it is indispensable during ontogenesis and involved in inflammation processes.[116] Activation by synthetic ligands results in decreased plasma triglycerides and increased HDL, which mirrors its stimulating effect on fatty acid oxidation in various tissues. Moreover, synthetic PPARδ agonist lead to upregulation of Abca1 in macrophages and other peripheral cells and thereby enhances reverse cholesterol transport.[117]

PPARγ is predominantly expressed in adipose tissue and of crucial importance for the differentiation to adipocytes.[118] In adipocytes, it is involved in the regulation of a wide variety of genes, many of them encoding for lipogenic genes. Based on these effects in adipocytes, PPARγ enhances fat storage. Furthermore, PPARγ activation increases insulin sensitivity: thiazolidinediones (TZDs), drugs widely used in diabetic patients, are synthetic ligands for PPARγ (see ref. 119 for review).

The liver receptor homolog 1 LRH-1

The liver receptor homolog 1 (LRH-1, NR5A2) does not form heterodimers with RXR, but binds to DNA as a monomer. It is expressed mainly in the liver, intestine, exocrine pancreas, and ovary. LRH-1 does not require a ligand but is constitutively active (see Fayard *et al*.[120] for review). Its expression starts already early during ontogenesis and LRH-1 knockout mice die around day 7 of embryonic development. Besides its important role in development, it is becoming more and more clear that LRH-1 is also a mayor player in the regulation of cholesterol metabolism. Main targets of LRH-1 are *Cyp7a1*, *alpha1-fetoprotein*, *multidrug resistance protein 3* (*Mrp3*), *cholesteryl ester transfer protein* (*CTEP*) and others. Recently, a LHR-1 binding motif was identified in the intergenic promoter of Abcg5/Abcg8 which underlines the importance of LRH-1 in the regulation of cholesterol absorption and hepatobiliary cholesterol excretion.[82]

Other transcription factors involved in the regulation of cholesterol metabolism

Sterol regulatory element-binding proteins SREBP

The sterol regulatory element-binding proteins (SREBP) are transcription factors - not belonging to the nuclear receptor superfamily - which are heavily involved in the regulation of lipid homeostasis. Three SREBP isoforms are known, *i.e.*, SREBP-1A, SREBP-1C, and SREBP-2, all expressed in the liver. SREBP-1A and 1C are derived from a single gene. SREBPs contain basic helix-loop-helix-zip domains.[121] The SREBPs are integral membrane proteins of the ER and the nuclear envelope. When cells are depleted from cholesterol, SREBPs are proteolytically cleaved in a two-step process and escorted to the nucleus.[122,123] Cleavage of the SREBPs is controlled by the SREBP cleavage-activating protein (SCAP), a protein that is regulated by the level of sterols present.[124] Target genes activated by SREBP-2 are mainly genes involved in cholesterol synthesis, *e.g.*, *HMG-CoA reductase*, whereas those of SREBP-1c are mainly involved in fatty acid synthesis (*e.g.*, *fatty acid synthase*). SREBP-1a activates genes in both categories (see Horton *et al.*[125] for review).

Cholesterol trafficking in the body

Cholesterol is of crucial importance for all cells in the body, but dietary intake and cellular demands widely vary, *e.g.*, during development and different nutritional states. Therefore, considerable quantities of cholesterol are circulating in the body at all times to provide a steady supply to cells.[126] It should be noted, however, that the brain is independent from this supply but relies on *de novo* synthesis.[2] The liver plays a central role in the distribution of cholesterol and several distinct pathways can be distinguished: Flux from the liver to the periphery; flux from the periphery to the liver; flux from the intestine to the liver; hepatobiliary excretion (see Fig. 9). Consequently, cholesterol flow within hepatocytes, enterocytes, and peripheral cells as well as the transport routes in between various organs need to be discussed.

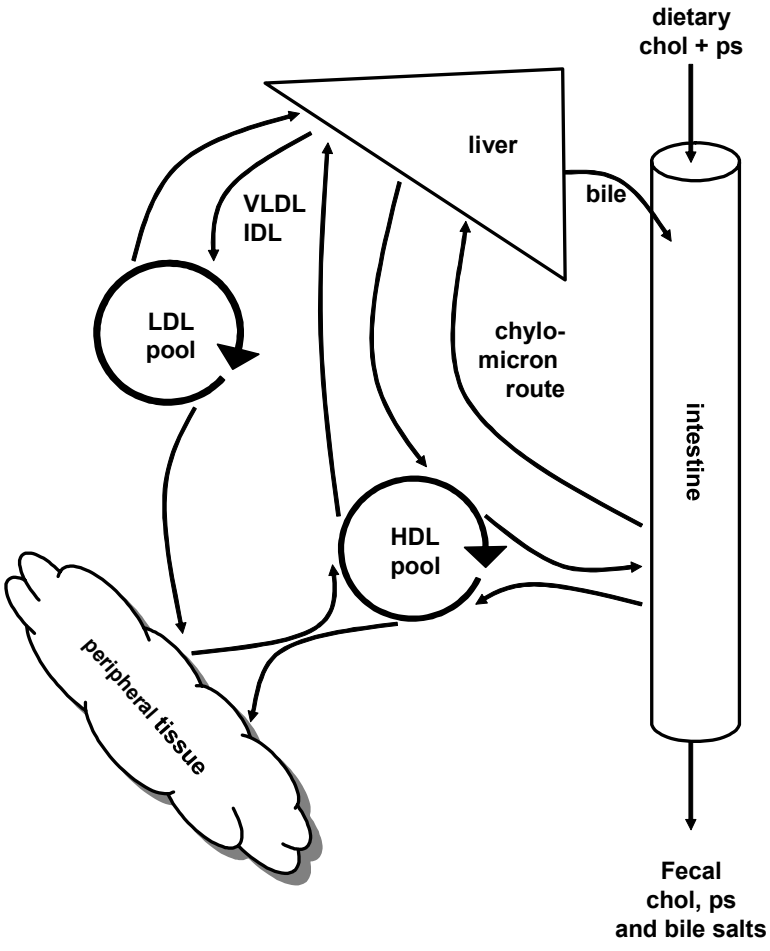


Figure 9: Schematic overview of the major routes of cholesterol in the human body.

Chol, cholesterol; HDL, high-density-lipoprotein; IDL, intermediate-density-lipoprotein; LDL, low-density-lipoprotein; ps, plant sterols; VLDL, very-low-density-lipoprotein.

Cholesterol flux in the enterocyte

About 50 % of dietary cholesterol is absorbed in the small intestine, with large individual variations (Fig. 10). In contrast, only small amounts of plant sterols are absorbed.[127,128] It has long been controversially discussed whether a protein is necessarily involved in intestinal cholesterol uptake. Recently, it was demonstrated that the Niemann-Pick C1-like protein 1 (Npc1l1) is of crucial importance for cholesterol absorption. In *Npc1l1* knockout mice, fractional cholesterol absorption is reduced from 51 to 16 %.[129] In parallel, plant sterol absorption is also reduced, indicating that Npc1l1 does not discriminate plant sterols from cholesterol, which points at downstream events as the main "discriminator" for plant sterol absorption.[130] Npc1l1 is likely the target of the new cholesterol absorption inhibitor ezetimibe. However, no direct interaction of ezetimibe to Npc1l1 has been demonstrated so far.[129] Ezetimibe has also been shown to target a complex formed from annexin 2 and caveolin 1 in enterocytes.[131] The relationship between the annexin 2-caveolin 1 route and Npc1l1 needs further investigation.

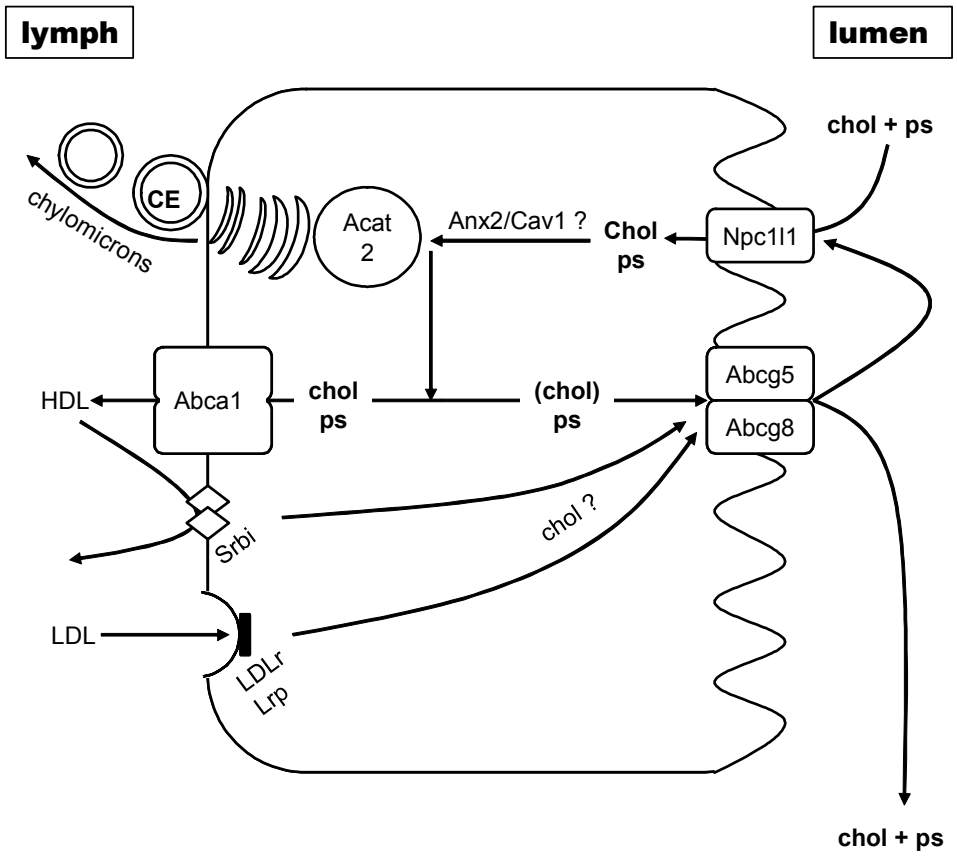


Figure 10: Schematic overview of the major routes of cholesterol in enterocytes.

Abca1, Abcg5, Abcg8, Abc-transporter a1, g5, g8; Acat, acyl-coenzyme A:cholesterol acyltransferase; Anx2/Cav1, annexin 2/caveolin 1; CE, cholesteryl ester; chol, cholesterol; HDL, high-density-lipoprotein; LDL, low-density-lipoprotein; LDLr, LDL receptor; Lrp, LDL-receptor related protein; Npc1l1, Niemann-Pick C1-like protein 1; ps, plant sterols; Srbi, scavenger receptor BI.

After uptake by the enterocyte, cholesterol is mainly esterified at C3 with fatty acids to form cholesteryl ester, a reaction catalyzed by acyl-coenzyme A:cholesterol acyltransferase 2 (Acat2).[132] Plant sterols are poor substrates for Acat2, thus the majority remains unesterified; this reflects a main difference between plant sterols and cholesterol for all reactions taking place in the enterocyte.[133,134] Cholesteryl esters can subsequently be secreted to the lymph after their packaging into chylomicrons and ultimately reach the liver (see below). The unesterified plant sterols are excreted back to the intestinal lumen, a process which is facilitated by the ABC-half transporters Abcg5 and Abcg8.[76,79] In addition, overexpression of Abcg5 and Abcg8 enhances excretion of cholesterol to the intestinal lumen and thereby limits net cholesterol absorption under these peculiar circumstances.[80,81,83]

Abca1 and ApoA-I are also expressed in enterocytes.[40,135] Hence, enterocytes are, in this respect, peripheral cells which can form HDL by transferring cholesterol (and possibly plant sterols) to lipid poor pre-ß-HDL. *Abca1*$^{-/-}$ mice do not show overtly reduced cholesterol absorption, which indicates that this process is probably less important for cholesterol absorption than chylomicron formation.[19,136,137]

In two studies (Chapters 2 and 5) described in this thesis, we demonstrate that the enterocyte under certain circumstances can transport cholesterol from the plasma compartment to the intestinal lumen. The last step, excretion to the lumen, can be explained by the action of Abcg5/Abcg8.[76,79] For this to happen, the enterocyte must be able to take up cholesterol from the plasma compartment. The LDL receptor has been shown to be expressed in enterocytes,[135] which makes receptor-mediated endocytosis a possible route. In addition, scavenger receptors, *e.g.*, SR-BI or CD36, are also potential candidates, but further study is needed to unravel the molecular mechanism behind this process.

Transport in the lymph: chylomicrons

The main excretory pathway of absorbed cholesterol from the enterocyte is provided by the chylomicrons (see ref. 138 for review). Chylomicrons are large, triglyceride-rich particles. The core of triglycerides and cholesteryl esters is surrounded by a phospholipid monolayer which also contains unesterified cholesterol and apolipoproteins (apoB48, apoA-I, apo A-IV). Chylomicrons are secreted into the lymph. When they reach the blood compartment, additional apolipoproteins are added (apoE, apoC-1, C-2, C-3) and the triglycerides are hydrolyzed by lipoprotein lipase to yield free fatty acids. Fatty acids are taken up by peripheral tissues (*e.g.*, muscle, adipose tissue) and some cholesterol ester is gained from HDL in exchange for triglycerides. In humans, this transport is mediated by the *cholesteryl ester transfer protein* (CETP).[139,140] The cholesterol-enriched chylomicron remnants are cleared by the liver, a process mediated by binding of apoE to the LDL-receptor (LDLR) or the LDL-receptor related protein (LRP).[141-143] Thus, virtually all cholesterol that is taken up from the intestine finally ends up in the liver.

Cholesterol flux in the hepatocyte

The liver is the central organ for cholesterol homeostasis and therefore the point of intersection of its various metabolic pathways (Fig. 11). The liver takes up cholesterol and cholesteryl esters from chylomicron remnants, IDL and LDL by receptor-mediated endocytosis by both the LDL-receptor and the LDL-receptor related protein.[141-143] The scavenger receptor SR-BI is responsible for the selective uptake of cholesterol from HDL particles without internalization of the complete particle (see ref. 144 for review). Moreover, a considerable amount of cholesterol synthesis takes place in the liver of most mammals. This cholesterol also has to be distributed to the target organs.

Inside the hepatocyte, cholesterol can be stored as cholesteryl ester after esterification by acyl-coenzyme A:cholesterol acyltransferase (Acat), secreted into bile as unesterified cholesterol, secreted into the blood stream - either as cholesteryl ester or as unesterified cholesterol - via the VLDL or HDL route, or be converted to bile salts and undergo hepatobiliary secretion. Two genes encoding Acat enzymes exist in mammals which differ in tissue distribution (see Cheng *et al.*[132] for review). In humans, ACAT1 is expressed in macrophages and hepatocytes (amongst other tissues), whereas ACAT2 is predominantly expressed in enterocytes. In

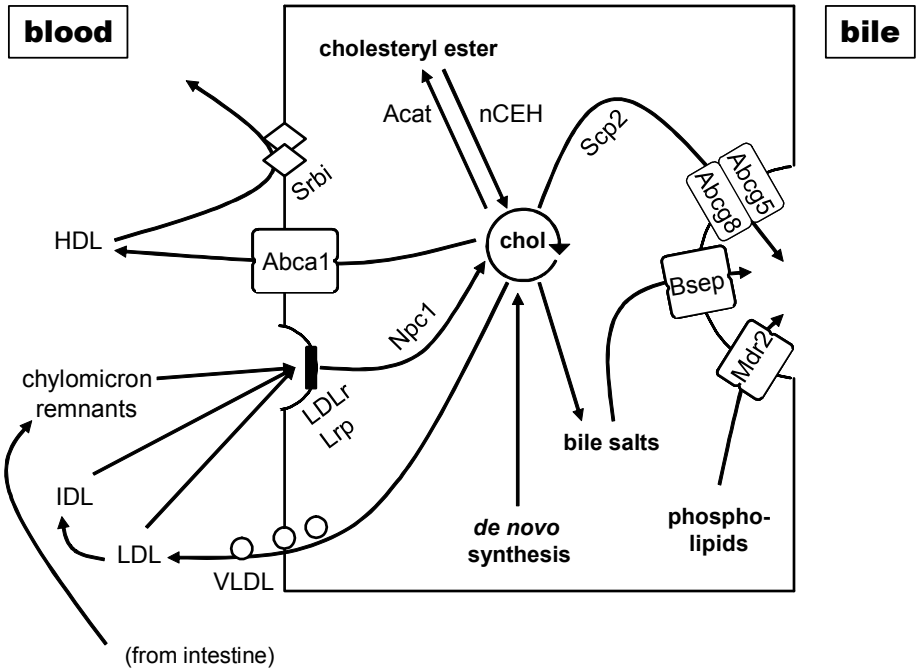


Figure 11: Schematic overview of the major routes of cholesterol in hepatocytes.

Abca1, Abcg5, Abcg8, Abc-transporter a1, g5, g8; Acat, acyl-coenzyme A:cholesterol acyltransferase; Bsep, bile salt export pump; chol, cholesterol; HDL, high-density-lipoprotein; IDL, intermediate-density-lipoprotein; LDL, low-density-lipoprotein; LDLr, LDL receptor; Lrp, LDL-receptor related protein; Mdr2, multidrug resistance P-glycoprotein 2; nCEH, neutral cholesteryl ester hydrolase; Npc1, Niemann-Pick C1 protein; Scp2, sterol carrier protein 2; Srbi, scavenger receptor BI; VLDL, very-low-density-lipoprotein.

mice, Acat2 is also highly expressed in the liver. Acat enzymes are crucial for storage of cholesterol as cholesterol ester. Re-mobilization of cholesterol requires hydrolysis by neutral cholesteryl ester hydrolase (nCEH).

Excretion to the systemic circulation of both cholesterol and cholesteryl esters occurs via two apolipoprotein-dependent routes: The VLDL pathway (see below) and the HDL pathway (see above). Also the synthesis of bile salts from cholesterol is discussed separately (see above). Bile formation and biliary excretion of bile salts and cholesterol is described below.

The intracellular routing of cholesterol in hepatocytes is poorly understood. However, some pathways putatively involved in intracellular routing have recently been deciphered (see ref. 145 for review). Studies in knockout- and transgenic mice indicate that, for example, the Niemann-Pick-C1 protein (Npc1)[146] and sterol carrier protein 1 (Scp2)[147] are involved in shuttling of cholesterol from the basolateral to the canalicular side of hepatocytes. Also the mRNA for the recently discovered intestinal cholesterol-uptake protein, *Npc1l1*,[129] is present in mouse liver, although at low concentrations. It is slightly upregulated in *Abcg5*$^{-/-}$ mice (unpublished results, see Chapter 4), a finding which cannot be interpreted without more detailed knowledge on its function in the liver.

Bile formation and enterohepatic circulation

Bile salt secretion is the major driving force for bile flow. Hepatocytes excrete bile salts to bile predominantly via the bile salt export pump (Bsep/Abcb11).[61] Water is passively following the osmotic gradient, a process which is most important for the generation of bile flow. Phospholipid and cholesterol excretion have been demonstrated to be coupled.[56] Phospholipids are translocated to bile by Mdr2/MDR3 (Abcb4), a process which can be stimulated by raising the bile salt flux through the hepatocyte.[54,57] The Abcg5/Abcg8 half transporters are involved in transport of cholesterol to bile; however, it is currently unknown *what precisely* they do. Overexpression of *Abcg5/Abcg8* by pharmacological means,[19,81] recombinant viral vectors[148] or in transgenic animals[80] leads to increased hepatobiliary sterol excretion. Knocking out both genes simultaneously virtually abolishes hepatobiliary cholesterol excretion,[85] and a strong correlation has been demonstrated between Abcg5/Abcg8 expression level and hepatobiliary cholesterol excretion rates in various animal models.[149] However, knocking out either one of the two genes does not give the results to be anticipated on the commonly accepted heterodimer model of Abcg5/abcg8 protein function.[83-85]

In the intestinal tract, both bile salts and cholesterol may undergo bacterial trans-formation.[24] Bacterial cholesterol metabolites are excreted with the feces. In contrast, cholesterol and bile salts (both primary and secondary) are, for a substantial part, taken up in the intestine and therefore re-enter the body. This cycling is referred to as *enterohepatic circulation*.

Transport to the periphery: VLDL/IDL/LDL

The Very-Low-Density-Lipoprotein (VLDL) particle is, in quantitative terms, probably the major shuttle for cholesterol from the liver to peripheral tissues. It is formed in the hepatocyte by lipidation of apoB100, a process mediated by the microsomal triacylglycerol transfer protein (MTP), and subsequent fusion with lipid particles. In detail, the newly formed apoB protein is translocated via the membrane of the endoplasmic reticulum. During translocation, apoB100 is folded and lipidated with phospholipids and some triglycerides by the action of MTP. Subsequently, it gathers lipid droplets present in the endoplasmic reticulum and/or the Golgi compartment and thereby collect the majority of its lipids. At the end, it consists of a cholesteryl ester/triglyceride core, surrounded by a phospholipid/cholesterol layer which is stabilized by a single apoB100 molecule (see ref. 150 for review).

Upon entering the blood, apoE and apoC-1, C-2, C-3 are added to the VLDL particle. Hydrolysis of triglycerides by LPL (as for chylomicrons) leads to formation of Intermediate-Density-Lipoprotein (IDL). IDL can be further hydrolyzed to Low-density-Lipoprotein (LDL) or be cleared by the liver. LDL is finally taken up by the liver or by peripheral cells, including macrophages, mediated by the LDL-receptor.[141]

Cholesterol flux in the peripheral cell

Peripheral cells, in contrast to hepatocytes and enterocytes, are limited in the number of cholesterol excretion pathways: they can only excrete cholesterol to the blood stream or lymph. The HDL-mediated transport of cholesterol from the periphery to the liver is usually referred to as *reverse cholesterol transport* (RCT) as described below in more detail. As the first step of reverse cholesterol transport, cholesterol and phospholipids are transferred to lipid-poor HDL particles via the action of Abca1.[42] This process is enhanced upon LXR activation (see above), thereby increasing removal of cholesterol from the cell.[46-48]

A second, Abca1-independent has recently been proposed: Abcg1, at least in macrophages, is thought to transfer cholesterol and phospholipids to mature HDL particles.[90] As the majority of plasma HDL is in the form of mature HDL, this would be an important new route for cholesterol disposal. Furthermore, Abcg4 could play an analogous role in the brain where it is highly expressed.[89]

Uptake of cholesterol and cholesterol esters in peripheral tissues is mediated by both the LDL receptor and scavenger receptors.[141,144] As exemplified above for the hepatocyte, Acat and CEH are involved in synthesis of cholesteryl esters for storage and hydrolysis for mobilization, respectively.[132]

Reverse cholesterol transport and HDL

The retrograde flux of cholesterol from peripheral cells to the liver (followed by fecal excretion), is called *reverse cholesterol transport* or *centripetal cholesterol flux*.[17,151,152]

HDL is formed by transfer of cholesterol and phospholipid to lipid poor HDL particles (pre-ß-HDL) mediated by Abca1 (see Attie *et al.* [153] for review). ApoA-I is the most prominent protein component of HDL. Cholesterol in the HDL particle is esterified by the action of *lecithin:cholesterol acyltransferase* (LCAT), resulting in cholesteryl ester-rich, mature HDL particles. In humans, cholesteryl esters are transferred to triglyceride-rich particles, *e.g.*, LDL particles, in a process mediated by CETP. In exchange, the HDL particle receives triglycerides which are subsequently hydrolyzed. In this way, a cholesterol-poor particle is regenerated which can function as a cholesterol acceptor in the periphery again.[139,140] An alternative fate for the mature HDL particle is binding to scavenger receptor BI (SR-BI) at the membrane of the target cell, followed by transfer of cholesterol and cholesteryl esters to the accepting plasma membrane by selective uptake.[154]

Removal of cholesterol from macrophages is considered to be beneficial against the development of lipid-loaded foam cells, the precursor of atherosclerotic lesions. Nevertheless, the contribution of macrophages to overall HDL formation is quantitatively a minor one.[155] The two components necessary for HDL formation, apoA-I and Abca1, are present in many different tissues, furthermore is a large pool of pre-ß- HDL particles available which is continuously secreted by the liver.[156] Hence, also hepatocytes and enterocytes contribute to overall HDL formation. The level of HDL is not limiting for reverse cholesterol transport *in vivo* (*e.g.*, refs. 17-19).

Outline and aim

Cholesterol is of crucial importance for a variety of physiological functions in mammals, *i.e.*, as a membrane constituent and as a precursor of bile salts and steroid hormones. However, one of its most prominent properties, its hydrophobicity, also makes its handling and metabolism difficult in the aqueous environment of the cell and establishes its role as a potential threat for health. Consequently, the level of cholesterol in the cell, determined by cholesterol synthesis, uptake and disposal, is subject of stringent regulatory mechanisms. Subsequently, these processes determine the level of the various cholesterol-containing lipoproteins in plasma and thus the risk for developing atherosclerosis.

For many decades, investigations focused on the way cholesterol is synthesized, transported in the blood, and converted to bile acids and other metabolites. Moreover, the involvement of transport proteins - and particularly ABC-transporters – in excreting cholesterol or bile salts from cells was of major interest, together with the various mechanisms of how cells can take them up again. During the last couple of years, it became evident that lipids or lipid derivatives may act as ligands for nuclear receptors and thereby have a large impact on the regulation of gene expression. A bouquet of synthetic and (putatively) physiological ligands has been described for many of these nuclear receptors, together with the evaluation of their stimulatory or inhibitory effects on gene expression.

The availability of agonist and antagonist of nuclear receptors involved in the regulation of lipid metabolism, in parallel with the construction of knockout mouse models for both nuclear receptors and (ABC)-transporters, stimulated – or even provoked – research on their interactions *in vivo*. Thus, aim of the research described in this thesis was to unravel the function of transport proteins potentially involved in cholesterol transport, their molecular regulation by nuclear receptors, particularly by LXR, and their physiological significance.

Abca1, the "Tangier disease protein", is responsible for the formation of HDL. Activation of LXR leads to increased expression of Abca1, which in turn increases plasma HDL cholesterol levels in various animal models. Accordingly, *Abca1*$^{-/-}$ mice do not possess HDL. HDL is generally considered the "big player" in reverse cholesterol transport and the major source for biliary cholesterol. In Chapter 2, we treated wild type and *Abca1*$^{-/-}$ mice with the synthetic LXR agonist T0901317 and studied its influence on hepatobiliary and fecal cholesterol excretion.

Two other targets of LXR are the half transporters Abcg5 and Abcg8. They putatively act as heterodimers to limit plant sterol absorption in the intestine and to excrete sterols from the hepatocyte to bile. Mutations in either one of them lead to accumulation of plant sterols in humans, a disease called *sitosterolemia*. We studied sterol metabolism in mice lacking functional Abcg5 (*Abcg5*$^{-/-}$ mice). Focus of the research described here was the function of Abcg5 in sterol absorption (Chapter 3) and sterol excretion (Chapter 4).

Classically, hepatobiliary excretion is considered as the most important route of cholesterol disposal for the body. In two LXR-activated models, however, we calculated that also the enterocyte significantly contributes to the cholesterol found in the feces. As this was only indirect evidence, we examined a model where hepatobiliary cholesterol excretion is absent, the *Mdr2*$^{-/-}$ mouse. *Mdr2*$^{-/-}$ mice do not excrete phospholipids nor cholesterol to bile. Consequently, all cholesterol found in the feces must be derived from the diet or from enterocytes. To evaluate this process, *Mdr2*$^{-/-}$ mice were treated with the LXR agonist GW3965 and transport of radio-labeled cholesterol from the plasma compartment to the feces was studied (Chapter 5).

Under pathophysiological conditions, hepatobiliary cholesterol excretion may be disturbed. This was found to be the case in the rare inherited disease *erythropietic protoporphyria* (EPP), which is a disorder of heme synthesis. In a mouse model of EPP, we describe the presence of an atypical lipoprotein, lipoprotein X (LP-X), in plasma (Chapter 6). We speculate that LP-X originates from bile-destined lipids which are secreted in a retrograde way.

The current knowledge on the involvement of ABC-transporters in cholesterol transport, both in the intestine and in the liver, is summarized in Chapter 7. Finally, the results obtained in this thesis are discussed and a perspective for future projects is presented.

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