SUMMARY

Cholesterol is essential for various physiological functions in mammals. It is a structural component of membranes and is thus important for normal cell functioning. Moreover, cholesterol is a precursor of biologically important compounds such as steroid hormones and bile acids. Because of its crucial importance, virtually all cells have the capacity for *de novo* synthesis of cholesterol. However, excess cholesterol can have deleterious effects for individual cells as well as the organism as a whole. Therefore, extensive control of cholesterol synthesis, catabolism, transport and excretion is required. Elevated plasma cholesterol, particularly LDL-cholesterol, constitutes an important risk factor for development of cardiovascular disease (CVD). On the other hand, high plasma levels of HDL are associated with protection against the development of atherosclerosis. This protective effect is ascribed, in part, to its capacity to transport cholesterol from peripheral tissues and vascular macrophages towards the liver for subsequent elimination via the feces, a process referred to as reverse cholesterol transport (RCT). In this “classical” concept of reverse cholesterol transport, the liver is often referred to as being the only organ capable to eliminate cholesterol via excretion into bile. However, recent studies have indicated that the intestine is an important secretory organ for cholesterol as well.

During the last decade, it has become clear that a number of nuclear receptors control expression of transporters crucial for cellular excretion of cholesterol. The research described in this thesis aimed to unravel the role of some nuclear receptors, specifically PPARγ, RXR and LXR, in control of various components of the reverse cholesterol transport pathway in vivo in mice. An overview of the processes involved in the maintenance of cholesterol homeostasis is provided in chapter 1.

First, we investigated the role of peroxisome proliferator-activated receptor (PPAR)γ in control of RCT. PPARγ activation is known to increase plasma HDL-cholesterol levels in primates and to stimulate apoA-I-specific cholesterol efflux from, as well as expression of ABCA1 in THP-1 macrophages. In chapter 2, we addressed the questions whether pharmacological activation of PPARγ in mice enhances reverse cholesterol transport and whether PPARγ-mediated effects require the presence of Abca1. Therefore, we treated both wild-type and Abca1<sup>−/−</sup> mice with a synthetic PPARγ agonist and determined the effects on cholesterol metabolism in detail. This study showed that activation of PPARγ led to elevated plasma HDL-cholesterol levels and increased expression levels of cholesterol transporters in peritoneal macrophages, suggesting an atheroprotective role of PPARγ. Moreover, PPARγ activation led to reduced expression of NPC1L1 in the intestine, coinciding with a 40% reduction in fractional intestinal cholesterol absorption. These data suggest that PPARγ is a promising target for development of novel drugs aimed at prevention of atherosclerosis.

RXR is the second nuclear receptor of which the role in control of RCT was assessed. In chapter 3, we investigated the effect of bexarotene, a synthetic RXR agonist, on atherosclerosis progression in a dyslipidemic murine model, the human apolipoprotein E2 knockin (KI) mouse, that develops lesions with cholesterol-laden macrophages. Since accumulation of cholesterol esters in macrophages in the arterial wall leading to transformation into foam cells is the hallmark of the atherosclerotic lesion, enhancement of macrophage efflux capacity is considered to be an attractive approach to diminish the risk for development of CVD.
Data from this study showed that bexarotene protects apoE2-KI mice from atherosclerotic lesion development, despite a marked increase in plasma triglycerides, likely by decreasing plasma levels of atherogenic cholesterol-containing lipoprotein fractions. The latter may be due to a marked decrease in fractional dietary cholesterol absorption through modulation of intestinal expression of Niemann-Pick-C1-Like1 (NPC1L1) and CD13. Moreover, bexarotene treatment only modestly modulates inflammatory gene expression in the vascular wall but markedly enhances the capacity of macrophages to efflux cellular lipids. Together, these data provide evidence for a favorable pharmacological effect of bexarotene on atherosclerosis development despite the induction of hypertriglyceridemia.

The third nuclear receptor that has been studied is LXR, which is a major player in regulation of cholesterol metabolism. This nuclear receptor regulates expression of several genes theoretically involved in RCT and activation of LXR by a synthetic agonist leads to elevated plasma HDL-cholesterol levels, reduced intestinal cholesterol absorption, increased hepatobiliary cholesterol secretion and increased sterol loss via the feces in rodent models. However, besides these beneficial effects, pharmacological LXR activation also increases lipogenesis and hence leads to hepatic steatosis. In Chapter 4, we evaluated the physiological role of LXR in control of both cholesterol transport and lipogenesis, in particular VLDL-TG production. For this purpose, we stimulated LXR by physiological means, i.e., by dietary cholesterol overload, in wild-type mice as well as in mice lacking the nuclear receptor Lxr.

On the high cholesterol diet, fractional cholesterol absorption was higher in Lxr\(^{-}\) mice than in controls leading to delivery of more dietary cholesterol to the liver. Lxr\(^{-}\) mice were not able to induce expression of hepatic Abcg5/Abcg8 and massive accumulation of free cholesterol and cholesteryl esters occurred. Hepatic cholesterol accumulation in Lxr\(^{-}\) mice was accompanied by lower hepatic triglyceride contents, strongly reduced plasma triglyceride concentrations (-90%), reduced VLDL-triglyceride production rates (-60%) due to displacement of TG by CE in newly formed VLDL particles and decreased hepatic expression of lipogenic genes, probably caused by impaired SREBP1c processing. The enhanced use of fatty acids for incorporation into cholesteryl esters together with the inability to induce lipogenesis resulted in a decreased VLDL-TG production and the formation of atherogenic, cholesterol-enriched VLDL particles. The unaffected VLDL-TG production in Lxr\(^{-}\)-deficient mice under chow-fed conditions indicates that LXR only has a direct effect in controlling VLDL composition under conditions of hepatocellular cholesterol overload.

Furthermore, in contrast to pharmacological activation of LXR\(_{α}\) by T0901317, activation of LXR\(_{α}\) by dietary sterols did not induce the full lipogenic gene repertoire or VLDL-TG production in wild-type mice, indicating differential effects of physiological and pharmacological LXR activation.

In Chapter 5, we focused on one specific component of RCT that is induced by LXR activation, namely hepatobiliary cholesterol secretion. The ABC half-transporters Abcg5 and Abcg8 are crucial for hepatobiliary cholesterol secretion and are both well-known targets of LXR. In this chapter, the function of this heterodimer in secretion of cholesterol was evaluated using a mouse model that lacks Abcg5. For this purpose, cholesterol secretion rates were measured in Abcg5\(^{-}\), Abcg5\(^{-}\) and wild-type mice. While bile flow and bile acid output were unaffected in Abcg5\(^{-}\) mice, cholesterol and phospholipid output were reduced by 85 and 31%, respectively. To get insight in the maximal cholesterol secretion capacity, mice were infused with increasing concentrations of the hydrophilic bile acid...
tauroursodeoxycholate to stimulate bile flow. Cholesterol secretion increased only minimally upon infusion of bile acids in Abcg5−/− mice, whereas in heterozygous Abcg5+/− mice, cholesterol secretion rates completely recovered upon bile acid infusion to reach wild-type levels. Upon LXR activation, the maximal cholesterol- and phospholipid output rates were dramatically increased in wild-type mice, whereas there was no effect in Abcg5−/− mice. These data suggest that Abcg5 is indeed strongly involved in the control of hepatobiliary cholesterol excretion. However, LXR-independent routes may exist that may contribute to overall hepatobiliary cholesterol output. This is supported by findings in Lxra−/− mice: Lxra−/− mice that were fed a high cholesterol diet secreted similar amounts of cholesterol into their bile as their wild-type and heterozygous controls did, even though these mice were unable to increase hepatic expression of Abcg5/Abcg8 upon cholesterol feeding. Expression levels of these genes were ~4-fold increased in wild-type mice. These studies provide evidence that, under certain metabolic conditions, a parallel route for biliary cholesterol secretion might be operational that is independent of Abcg5/Abcg8.

The pathway of hepatobiliary cholesterol secretion was further investigated in chapter 6. Several processes involved in cholesterol metabolism, e.g., de novo cholesterol synthesis and bile acid formation, are fluctuating during the day. Since biliary secretion of cholesterol is strongly associated with bile acid secretion, we addressed the question whether expression of Abcg5 and Abcg8 and hence the hepatobiliary secretion of cholesterol also displays day-night variation. Because the biological clock “output” transcription factor DBP is involved in the diurnal expression of Cyp7a1 and various aspects of cholesterol metabolism are coordinately regulated, we also assessed whether DBP is involved in control of expression of Abcg5 and Abcg8. Data from this study showed that hepatic expression of Abcg5 and Abcg8 displayed a circadian pattern similar to that of the Hmgr gene in mice, with a maximum late in the afternoon (ZT8-12; ZT0 is light-on) and a nadir (~70% compared to ZT8-12) early in the morning (ZT20-24). High Abcg5/Abcg8 expression levels in the late afternoon were associated with a two-fold higher hepatobiliary secretion rate of cholesterol at this time-point compared to early morning. Dbp−/− mice showed higher expression levels of Abcg5/Abcg8 in the liver during the complete day and the circadian fluctuations were clearly amplified when compared to controls. Overall induced Abcg5/g8 expression led to a 50% increase in biliary cholesterol secretion, measured between ZT8-10, in Dbp−/− mice. However, expression of Abcg5 and Abcg8 appeared to be not directly transcriptionally regulated by DBP.

Hepatobiliary secretion of free cholesterol is often referred to as a main route for elimination of cholesterol from the body. However, recent studies have proposed the intestine to be a quantitatively important secretory organ for cholesterol as well. The fractional contribution of this pathway, that may represent an arm of the reverse cholesterol transport pathway, has not been directly established so far in vivo. In the work described chapter 7, we have developed a method to quantify the fractional and absolute contributions of several cholesterol fluxes to total fecal neutral sterol loss in vivo in mice, by assessing the kinetics of orally and intravenously administered ²H- and ¹³C-labeled cholesterol combined with an isotopic approach to assess the fate of de novo synthesized cholesterol. Our results showed that direct intestinal cholesterol secretion significantly contributes to removal of blood-derived free cholesterol in C57Bl6/J mice (33% of 231 µmol/kg/day) and that pharmacological LXR activation with T0901317 strongly stimulates this pathway (63% of 706 µmol/kg/day). Direct intestinal cholesterol secretion is impaired in mice lacking Abcg5 (-64%
compared to wild-type controls), suggesting that the Abcg5/Abcg8 heterodimer is involved in this pathway. Data from this study demonstrate that intestinal secretion represents a quantitatively important route for fecal removal of neutral sterols independent of biliary secretion in mice and that pharmacological LXR activation strongly stimulates this new arm of the reverse cholesterol transport pathway. These data therefore strengthen the concept that the intestine might contribute substantially to reverse cholesterol transport. Activation of LXR specifically aimed at the intestine could thus be an attractive approach to stimulate reverse cholesterol transport without inducing undesirable systemic side-effects, such as hepatic steatosis.

Thus, agonists for the nuclear receptors PPARδ, RXR and LXRα have been shown to interfere with several components of reverse cholesterol transport and are therefore considered as promising tools for the development of novel drugs aimed at preventing atherosclerosis. Overall, the studies described in this thesis allow the conclusion that the intestine may be a promising target organ for nuclear receptor interference in order to stimulate reverse cholesterol transport. In addition to interference with fractional intestinal cholesterol absorption, nuclear receptor activation may modulate direct intestinal cholesterol excretion independent of biliary cholesterol secretion and could therefore be an attractive approach to develop novel drugs for treatment or prevention of atherosclerotic cardiovascular diseases.