Novel pathways in cholesterol metabolism to combat cardiometabolic diseases

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Summary

Atherosclerotic cardiovascular disease (CVD) and non-alcoholic fatty liver disease (NAFLD), which can develop into nonalcoholic steatohepatitis (NASH), are both cardiometabolic diseases. Atherosclerotic CVD is the leading cause of death globally, whereas current therapeutic pharmacological approaches only prevent 25-45% of cardiovascular events. NAFLD/NASH is one of the most common chronic liver diseases worldwide, but approved medications are even not yet available. Since hypercholesterolemia and inflammation are common major risk factors for atherosclerotic CVD as well as NAFLD, new therapeutic strategies to combat these cardiometabolic diseases by lowering cholesterol and inflammation are eagerly awaited. The studies described in this thesis thus aimed to get insight in strategies how to further improve cholesterol metabolism and inflammation, by exploring the therapeutic potential of brown fat activation and transcription factors involved in both processes.

Chapter 1 provides a general introduction of cholesterol metabolism and its regulation by transcription factors such as liver X receptors (LXR) and the farnesoid X receptor (FXR). Furthermore, the role of hypercholesterolemia in the etiology of atherosclerotic CVD and nonalcoholic steatohepatitis (NASH) is pointed out, current therapeutic strategies are outlined, and knowledge gaps are mentioned. Finally, brown fat and transcription factors as emerging targets for the treatment of these cardiometabolic diseases are introduced.

Previous studies in female *APOE*3-Leiden.CETP* (*E3L.CETP*) mice, a well-established model for human-like cardiometabolic disease, have shown that brown fat activation increases lipoprotein lipase (LPL) mediated fatty acid extraction from triglyceride-rich lipoproteins (TRL) such as very low-density lipoproteins (VLDL), thereby accelerating the hepatic uptake of cholesterol-enriched TRL remnants via the ApoE/LDL receptor (LDLR) clearance pathway and increasing high density lipoprotein (HDL) in the circulation. As such, brown fat activation lowers plasma cholesterol via hepatic LDLR and increases HDL-mediated reverse cholesterol transport via hepatic scavenger receptor class B type 1 (SRB1), collectively protecting against atherosclerosis development.

First, given that brown fat activation increases LPL-dependent generation of TRL remnants, we postulated that brown fat activation can increase the therapeutic effectiveness of proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibition that has recently emerged as a novel strategy to reduce atherosclerosis by upregulation the hepatic LDLR to lower (V)LDL-cholesterol. Therefore, in **Chapter 2**, we aimed to investigate whether accelerating lipolytic processing of VLDL by brown fat activation can further lower (V)LDL-cholesterol and reduce atherosclerosis on top of PCSK9 inhibition. To this end, female *E3L.CETP* mice were treated with the anti-PCSK9 antibody Alirocumab or saline as controls. After two weeks, both groups of mice were randomized to additionally receive either the selective β3-adrenergic receptor (β3-AR) agonist CL316,243 to activate brown fat or saline. β3-AR agonism and PCSK9 inhibition combined decreased (V)LDL-cholesterol compared to PCSK9 inhibition alone, which was explained by an accelerated plasma clearance of VLDL-cholesteryl esters that were mainly taken up by the liver. In addition, combination treatment promoted the transfer of VLDL-phospholipids to HDL to a higher extent than PCSK9 inhibition alone, accompanied by higher plasma HDL-cholesterol levels and increased cholesterol efflux capacity. Consequently, combination treatment largely reduced atherosclerotic lesion area compared to vehicle. Together, β3-AR agonism enhances the lipoprotein-modulating effects of PCSK9 inhibition to further improve dyslipidemia and non-significantly further attenuate atherosclerosis development in the aortic root. The findings presented in this Chapter thus demonstrate that brown fat activation may enhance the therapeutic effects of PCSK9 inhibition in dyslipidemia.

As explained above, besides the LDLR, we hypothesized that hepatic SRB1 may also mediate the anti-atherogenic effects of brown fat activation by facilitating reverse cholesterol transport. To figure out the specific role of hepatic SRB1 in the atheroprotective properties of brown fat activation, in **Chapter 3** we used adeno-associated virus serotype 8 to specifically knock down hepatic SRB1 on top of brown fat activation by β3-AR agonism using CL316,243, again in female *E3L.CETP* mice. Surprisingly, hepatic SRB1 knockdown appeared to additively improve the beneficial effects of β3-AR agonism on atherosclerosis development. In fact, hepatic SRB1 knockdown *per se* not only increased HDL-cholesterol levels, but also reduced plasma triglyceride and non-HDL-cholesterol levels, thus explaining the reduction in atherosclerosis development. Mechanistic studies indicated that this is due to increased lipolytic processing and hepatic uptake of VLDL by facilitating VLDL-surface transfer to HDL. Taken together, we conclude that hepatic SRB1 knockdown, at least in a mouse model with an intact ApoE-LDLR clearance pathway, relevant to human lipoprotein metabolism, reduces atherosclerosis in the aortic root and improves the beneficial effect of brown fat activation on atherosclerosis development, which is explained by pleiotropic effects of hepatic SRB1 knockdown on lipolytic processing and hepatic uptake of VLDL. The results from this chapter thus reveal that brown fat activation could thus be an effective strategy to treat CVD also in subjects with impaired SRB1 function.

Brown fat activation thus largely increases the flux of cholesterol derived from TRL remnants and also HDL to the liver, which was already shown to increase the hepatic conversion of cholesterol into bile acids (BA) that are secreted via the bile into the intestine. Upon short term brown fat activation, a considerable fraction of BA escapes enterohepatic circulation and is excreted via the feces, explaining that acute brown fat activation increases reverse cholesterol transport. In **Chapter 4**, we showed that prolonged β3-AR agonism actually reduces fecal BA excretion, while markedly increasing plasma levels of total BAs with respect to both cholic acid-derived BAs and chenodeoxycholic acid-derived BAs, increasing liver cholesterol and decreasing the expression of genes involved in hepatic BA production. As we postulated that these effects were explained by adverse effects resulting from markedly stimulated enterohepatic circulation of BA, we next evaluated the effects inhibition of intestinal BA reabsorption on plasma cholesterol metabolism and atherosclerosis development combined with brown fat activation. To this end, female *E3L.CETP* mice again were treated for 9 weeks with the β3-AR agonist CL316,243 to activate brown fat without or with the BA sequestrant Colesevelam to inhibit BA reabsorption from the intestine. Concomitant intestinal BA sequestration increased fecal BA excretion, normalized plasma BA levels and reduced hepatic cholesterol. Moreover, BA sequestration on top of β3-AR agonism, as compared to β3-AR agonism alone further reduced plasma total cholesterol and non-HDL-cholesterol, tended to further attenuate atherosclerotic lesion area and further increased the proportion of lesion-free valves in the aortic root. Collectively, the data presented in this Chapter suggest that combining brown fat activation with BA sequestration is a promising new therapeutic strategy to reduce hyperlipidemia and CVD.

Besides brown fat activation, we next focused on the potential of the nuclear receptors LXR and FXR to improve cardiometabolic health. Since LXRs are key regulators of metabolic and inflammatory signaling, they have taken center stage as potential therapeutic targets for the treatment of cardiometabolic diseases. However, undesirable effects of pharmacological LXR activation, including hyperlipidemia and neutropenia have thus far prevented clinical application. Desmosterol, which is converted by Δ24-dehydrocholesterol reductase (DHCR24) into cholesterol, is an endogenous LXR ligand that does not induce lipogenic gene expression. In theory, increasing endogenous desmosterol levels by targeting DHCR24 is thus a promising

strategy for activating LXR transcription programs to combat atherosclerotic CVD as well as NAFLD/NASH. In **Chapter 5**, we first investigated the metabolic and immunological consequences as well as anti-inflammatory potential of increased desmosterol through DHCR24 inhibition, using the in-house potent and selective DHCR24 inhibitor SH42. We showed that SH42 activates LXR in macrophages, not by direct binding but highly dependent on endogenous cholesterol biosynthesis, and without inducing lipogenic gene expression. DHCR24 inhibition by SH42 increased plasma desmosterol levels and inflammation resolution, and decreased pro-inflammatory cell influx in a zymosan A-induced murine peritonitis model. Lipidomic and lipid mediator analysis further revealed that SH42 increased the biosynthesis of endogenous polyunsaturated fatty acids and the production of pro-resolving lipid mediators. Based on these findings, we provide a conceptually new strategy to induce LXR by desmosterol indirectly through DHCR24 inhibition, and expect such as strategy not to induce lipogenesis for the treatment of both inflammatory and cardiometabolic disease.

Therefore, in **Chapter 6** we next investigated whether DHCR24 inhibition to increase desmosterol and consequently activate LXR could be a therapeutic strategy for the treatment of NAFLD, a disease of metabolic inflammation in the liver. To this end, male *E3L.CETP* mice were fed a high fat and high cholesterol diet to develop diet-induced human-like NAFLD/NASH characteristics, with or without simultaneous treatment with SH42. After 8 weeks of treatment, we found that SH42 markedly increased liver and plasma desmosterol levels, reduced hepatic diacylglycerol and triacylglycerol levels as well as the hepatic steatosis score, while SH42 decreased plasma free fatty acid and cholesteryl ester concentrations. Flow cytometry analysis showed that SH42 decreased liver inflammation by preventing Kupffer cell activation and monocyte infiltration. The beneficial effects of SH42 on liver lipid levels, steatosis score and liver inflammation were complete abolished by LXRα-deficiency. Together, we conclude from these data that inhibition of DHCR24 by SH42 leads to an increase in desmosterol and prevents diet-induced hepatic steatosis and inflammation in a strictly LXRα-dependent manner without causing hyperlipidemia. We thus anticipate that pharmacological DHCR24 inhibition may represent a potential novel therapeutic strategy for treatment of NAFLD. If SH42 also appears effective in preventing atherosclerotic CVD, SH42 could present a true revolution in the treatment of cardiometabolic disease by targeting inflammation in macrophages, which would largely add to current strategies that mainly focus on lowering of lipids.

In addition to LXR, the intracellular BA receptor FXR is another promising therapeutic target for treatment of cardiometabolic diseases. Since the effects of pharmacological modulation of BA signaling and metabolism on dyslipidemia are incompletely understood, in **Chapter 7** we investigated the effects of FXR activation on various measures of cardiometabolic health. Male *E3L.CETP* mice were fed a Western-type diet for 8 weeks to induce metabolic syndrome before treatment with or without the non-steroidal FXR agonist PX20606 for 4 weeks. PX20606 treatment decreased plasma triglyceride and cholesterol levels, primarily due to reduction of ApoB-containing lipoproteins. Mechanistically, PX20606 treatment induced a more hydrophilic bile acid pool, which decreased intestinal cholesterol absorption and increased fecal cholesterol excretion. In addition, PX20606 treatment increased ApoC2/ApoB ratio, suggesting accelerated VLDL-triglyceride catabolism. Collectively, we showed that FXR stimulation strongly enhances fecal cholesterol disposal and corrects dyslipidemia. Although care should be taken when extrapolating these results to humans, particularly because of species-differences in BA metabolism, pharmacological modulation of BA metabolism via FXR activation may hold potential for the treatment of dyslipidemia-associated cardiometabolic diseases.

Finally, in **Chapter 8** we discussed the results of this thesis and the high potency of targeting brown fat and DHCR24 as novel therapeutic targets for the treatment of cardiometabolic diseases. Collectively, the studies described in this thesis have increased our insight into regulation of cholesterol metabolism and inflammation by brown fat and nuclear receptors, respectively, and provided promising leads for innovative treatment of cardiometabolic diseases including brown fat activation and DHCR24 inhibition by SH42.