

# Intervention in hepatic lipid metabolism : implications for atherosclerosis progression and regression

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# Chapter 8

### General discussion and perspectives

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#### **GENERAL DISCUSSION AND PERSPECTIVES**

Atherosclerosis is the underlying cause of most cardiovascular diseases<sup>1</sup>. Hypercholesterolemia holds a key role in the development of atherosclerosis and is a causative factor for coronary artery disease<sup>2</sup>. Lowering of VLDL- and LDL-cholesterol levels leads to a reduction in cardiovascular morbidity and mortality<sup>3</sup>. In contrast, high levels of HDL-cholesterol are associated with a decreased risk of cardiovascular disease<sup>4</sup>. This dissertation is dedicated to the regulation of lipid metabolism pathways and its subsequent effects on atherosclerotic lesion progression and regression.

#### 1. Hepatic lipid metabolism and pharmaceutical interventions in the liver

Atherosclerosis is a liver disease of the heart<sup>5</sup>. Liver is considered as the major organ with significant therapeutic importance for the maintenance of metabolic homeostasis<sup>6</sup>. The first part of the thesis focuses on the hepatic lipid metabolism and the pharmaceutical interventions in the liver.

## 1.1 Nuclear receptors in the liver: potential targets for pharmaceutical interventions

Nuclear receptors (NRs) are ligand-activated transcription factors that act as sensors for a broad range of natural and synthetic ligands. A growing number of NRs have been identified as regulators in lipid metabolism and energy utilization<sup>7</sup>. Moreover, NRs control hepatic inflammation, regeneration, fibrosis, and tumor formation. Therefore, NRs are crucial for understanding the pathogenesis and pathophysiology of a wide range of hepatic disorders. Targeting NRs and their regulations in the liver offers new perspectives for the treatment of metabolic diseases<sup>8</sup>.

In Chapter 2, we have composed the hepatic cell type-specific expression profile of NRs to provide the most complete quantitative assessment of NRs distribution in the liver reported to date. We have identified several liver-enriched orphan NRs as potential novel targets for pharmaceutical interventions. Specifically, members of the orphan receptor family COUP-TFs showed distinguished distributions in liver. COUP-TF3 was abundantly and exclusively expressed in liver parenchymal cells, while the other two members, COUP-TF1 and COUP-TF2, were expressed exclusively in non-parenchymal cells. Despite the moderate to high expression level in the liver, the physiological functions of COUP-TFs have not been fully exploited, and the ligand for COUP-TFs has not been identified. Our study suggests a physiologic important role of COUP-TF3 in hepatocytes, and that of COUP-TF2 in endothelial cells and macrophages. Previous studies have shown that the activity of COUP-TFs is associated with the transcriptional regulation of a number of genes expressed mainly in the liver<sup>9</sup>. COUP-TF2 and COUP-TF3 have been generally considered to be repressors or regulators for transcription of NRs such as RARs, TRs, PPARs, and HNF4alpha<sup>10</sup>. The cellular distribution pattern from the present study suggests that COUP-TF3, given its high expression in liver parenchymal cells, may have a potential role in hepatic lipid and xenobiotic metabolism regulation via cross-talking with other liver-enriched NRs. Our study identifies COUP-TF2 as highly expressed in endothelial cells, which is in

accordance with published data upon the role of COUP-TF2 in angiogenesis and generation of haematopoietic cell clusters<sup>11</sup>. In addition, COUP-TF2 was shown to have a potential role in regulation of cholesterol homeostasis<sup>12</sup>. The observation from our current study that the expression of COUP-TF2 is as high as NGFIB in Kupffer cells raises interest to further investigate whether COUP-TF2 is involved, similarly as NGFIB, in lipid metabolism in macrophages.

We used real-time quantitative PCR to gain the expression profiles of NRs. It is a standardized method in the NR field to characterize the expression pattern of individual receptors in tissue or cells. It provides a simple but powerful way to obtain comprehensive understanding of the distribution and biological functions of NRs<sup>13</sup>. Real-time quantitative PCR has been applied in numerous studies to profile the expression pattern of NRs in tissues representing diverse anatomical systems under various pharmacological conditions and genotypes<sup>14,15</sup>. However, because NRs are transcription factors, the regulation of NRs are not only at the mRNA level, but also on the protein activity level. Therefore, it is important to further extend the gene expression profiles of NRs into their protein expression and/or functionality profiles by using immunohistochemical staining or flow cytometry. Furthermore, in addition to the hepatic endothelial cells and Kupffer cells, which were investigated in the current study, hepatic stellate cells are also major repositories for lipids, being activated during chronic liver injury and mediating the fibrotic response<sup>16,17</sup>. It is thus interesting to include in future studies the hepatic stellate cells when investigating gene expression profiles of NRs in the liver.

#### 1.2 Niacin regulates hepatic lipid metabolism and CETP expression

The use of statins in patients with high risk for cardiovascular disease (CVD) has resulted in a 30-40% decrease in clinical events in the last couple of decades. However, despite of a marked reduction (up to 60%) in LDL-C, about 30% of patients continue to have CVD events<sup>18</sup>. Therefore, HDL has become the next promising therapeutic target. The anti-dyslipidemic drug niacin, also known as nicotinic acid, not only lowers plasma levels of pro-atherogenic lipids/lipoproteins but also is the most effective agent available to increase HDL-cholesterol<sup>20,21</sup>. Several clinical trials have shown that niacin reduces cardiovascular disease and myocardial infarction incidence, providing an emerging rationale for the use of niacin in the treatment of atherosclerosis<sup>22,23</sup>. Only recently, it has been shown that niacin also reduces the hepatic expression and plasma levels of the proatherogenic CETP<sup>24</sup>. In Chapter 4 we investigated the mechanism underlying the CETP-lowering effect of niacin. We propose that the primarily reduced hepatic cholesterol accumulation via the lipid-lowering effect of niacin leads to attenuated hepatic inflammation, and thus less macrophage infiltration into and/or increased macrophage emigration out of the liver. The decreased amount of hepatic macrophages, which are significant contributors of CETP, leads to an overall reduction in hepatic CETP expression and a lower plasma CETP level. In conclusion, we have shown that niacin does not directly alter macrophage CETP expression, but attenuates the liver inflammation and macrophage content in response to its primary lipid-lowering effect, which leads to a decrease in hepatic CETP expression and the plasma CETP mass. Our study sheds a new light on the mechanism underlying the CETP-lowering effect of niacin in CETP transgenic mice.

Niacin receptor GPR109A is highly expressed in adipose tissue. Interestingly, adipose tissue appears to be a highly conserved site of CETP expression across

species. Adipose tissue CETP makes a major contribution to CETP in the circulation, reduces HDL, and increases non-HDL cholesterol levels. Adipose tissue CETP plays a local role in adipocyte cholesteryl ester accumulation from HDL, indicating a novel role of CETP in modulating adiposity. It is thus of interest to further investigate the potential regulation effects of niacin on adipose tissue CETP expression.

Hernandez et al<sup>25</sup> demonstrated for the first time the critical role of CETP in niacin-mediated HDL-elevation by using CETP tg mice and suggested that transgenic mice expressing the human CETP could be a useful animal model for studying the established HDL-regulating effect of niacin. However, in the current study we did not observe the HDL-raising effect of niacin in CETP Tg mice. This might be due to diet differences. Hernandez et al used regular chow diet supplemented with niacin, while we used WTD diet which contains more fat and 0.25% cholesterol that has been shown to upregulate CETP expression. Furthermore, although niacin significantly increased hepatic apoA-I expression in our study, the hepatic expression of ABCA1, which is involved in apoA-I-mediated cholesterol efflux, significantly decreased by 45% at the same time upon niacin treatment, which can ultimately result in unchanged HDL formation in plasma. Interestingly, our observations corroborate recent data from Li et al<sup>26</sup>, who showed that niacin-mediated activation of GPR109A in liver led to reduced hepatocyte ABCA1 expression and thus an unexpectedly reduced plasma HDL-C level in C57BL/6J mice. The current study was set out to investigate the mechanism underlying the hepatic and plasma CETP-lowering effect of niacin in mice, instead of determining the mechanism of HDL-C elevation by niacin. This mechanistic approach in a mouse model thus differs from a clinical investigation.

Recent unexpected termination of AIM-HIGH Clinical Trial on niacin and HDL hypothesis has drawn much attention. AIM-HIGH is the first large randomized trial to evaluate the effect of niacin on cardiovascular events among statin-treated patients with established atherosclerotic cardiovascular disease who are at the desired level for LDL-C but who have residual abnormalities in HDL-C and triglycerides. It was meant to test whether adding high dose, extended-release niacin to a statin (simvastatin) is better than a statin alone in reducing long-term cardiovascular events in participants<sup>27</sup>. The trial was started in September 2005, but was stopped early due to the lack of incremental benefit on cardiovascular risk reduction in the extended-release niacin plus simvastatin treatment group over simvastatin alone. At this time, the FDA has made no new conclusions or recommendations regarding the use of extended-release niacin alone or in combination with simvastatin or other statins<sup>28</sup>. The lack of additional effect of niacin on cardiovascular events is unexpected and a striking contrast to the results of previous trials and observational studies, despite that niacin in this trial produced the predicted effects on all lipid parameters measured including increasing HDL levels by 20% and reducing triglycerides by around  $25\%^{29}$ . The early termination of this trial raised questions about the suggested beneficial effects of the HDLelevation hypothesis<sup>30</sup>. However, the AIM-HIGH study population was not a very high-risk population because the patients were already on statin-treatment and their LDL-cholesterol level was already well controlled. The study population in this trial does not represent all patient populations in whom the importance of treating low HDL and lowering triglycerides with Niaspan may be significant. Therefore, the relevance of these results to patients outside the AIM-HIGH study population is

currently unknown and it would be premature to extrapolate these results with Niaspan to a broader patient population at this time<sup>31</sup>.

The clinical use of niacin has been limited due to the cutaneous flushing effect, which is mediated by the niacin receptor GPR109A in the skin. Therefore, in Chapter 5, we assessed the properties of two partial agonists for GPR109A and compared them to niacin. We show that these two GPR109A partial agonists of the pyrazole class are promising drug candidates to achieve the beneficial lipidlowering effects while they successfully avoid the unwanted flushing side effect. Interestingly, our flushing results are somewhat complicated due to the unexpected skin temperature increase observed in the C57BL/6 mice treated with the vehicle DMSO. DMSO is one of the most common solvents used for in vivo administration of water-insoluble substances. In literatures, a wide range of pharmacological effects of DMSO has been documented in both animal and human experimental models, including membrane penetration and vasodilation<sup>32,33,34</sup>. It is proposed that DMSO provokes a histamine-like response, possesses potent histamine-liberating properties, and increases blood flow<sup>35</sup>. Despite being frequently used as a solvent in biological studies and as a vehicle for drug therapy, the undesirable side-effects of DMSO both in vivo and in vitro should be taken into consideration<sup>36</sup>.

#### 2. Atherosclerotic lesion regression and mouse models

The second part of the thesis focuses on the concept of atherosclerotic lesion regression, shedding insights in the role of LXR activation and application of mouse models in regression studies.

While numerous studies have been dedicated to inhibit the progression of atherosclerosis, recent attention has been focused on reversing atherosclerosis, which is the regression of existing atherosclerotic plaque. In previous studies, a dramatic regression of large advanced lesion was achieved with or without LXR agonist<sup>37,38</sup> by utilizing surgical aorta transplantation into wildtype mice. This procedure leads to a rapidly improved milieu for the atherosclerotic lesions. In another mouse model, apoE\*3Leiden mice, LXR agonist were also shown to promote regression of moderate lesions<sup>39</sup>. In both **Chapter 6** and **Chapter 7** we investigated other different mouse models to explore technically easier and more efficient approaches for plaque regression.

In **Chapter 6** we first fed LDLr<sup>-/-</sup> mice with high-fat high-cholesterol Westerntype diet (WTD) for 6 weeks to develop atherosclerotic plaques. Subsequently, a group of mice was sacrificed to obtain baseline data, whilst the rest of the mice were switched to a low-fat cholesterol-free chow diet without or with LXR agonist T0901317 supplementation for 3 weeks. Unexpectedly, T-0901317 supplemented in regular chow diet largely elevated plasma cholesterol level, and thus the atherosclerotic plaque continued its progression. Therefore, we set out to evaluate the effects of LXR agonist on plaque regression in C57BL/6 mice. The C57BL/6 mouse model has been used to study diet-induced atherosclerosis<sup>40,41,42</sup>. This murine model of atherogenesis represents an alternative to the use of genetically modified mice with impaired lipoprotein clearance, i.e. LDLr<sup>-/-</sup> mice, for the evaluation of anti-hyperlipidemic agents, including LXR agonists<sup>43</sup>. Here we fed C57BL/6 mice with cholate-containing cholesterol-enriched atherogenic diet for 16 weeks to induce atherosclerotic plaque development. Subsequently, a group of mice were sacrificed to obtain baseline data, whilst the remainder of the mice was

switched to low-fat cholesterol-free chow diet without or with LXR agonist T0901317 supplementation for 3 weeks. In contrast to data from  $LDL^{-/-}$  mice, in C57BL/6 mice LXR agonist supplemented in regular chow diet successfully reduced the diet-induced hypercholesterolemia rapidly. LXR agonist not only reduced plasma (V)LDL cholesterol levels, but also significantly increased HDL level, leading to a 10-fold increased ratio between antiatherogenic HDL and proatherogenic (V)LDL level. This indicates that LXR agonist, together with chow diet, rapidly improved the plasma lipoprotein profile and induced a regression environment in C57BL/6 mice.

LXR agonist exhibited opposite effects on plasma lipoprotein and lesion progression in LDLr<sup>-/-</sup> and C57BL/6 mice, and we propose that this is due to the absence of LDL receptor and thus impaired LDL clearance in LDLr<sup>-/-</sup> mice. The LDL receptor-mediated cholesterol uptake and feedback regulation are crucial when evaluating LDL-lowering effects of compounds<sup>44</sup>. Studies have show that when LDL receptor gene was transferred back into hypercholesterolemic LDLr<sup>-/-</sup> mice to restore sustained expression of the LDLr protein in the liver, there was drastic reduction of plasma cholesterol and non-HDL cholesterol levels persistently and pronounced regression of advanced atherosclerotic<sup>45,46</sup>. Thus, it is proposed that C57BL/6 mice is a better model than LDLr<sup>-/-</sup> to evaluate (V)LDL-lowering induced atherosclerotic regression.

However, the limitation of the C57BL/6 mouse model is that the atherosclerotic plaque induced by an atherogenic diet was relatively small (~50 x  $10^3 \ \mu m^2$  in the aortic root), and there was hardly any lesion developed in the aortic arch or the descending aorta yet. Thus, the effect of LXR-agonist in this study was limited to diminishing early atheromatous lesion with small size and less complexity. It is thus important to further investigate the potential of LXR activation on diminishing larger advanced atherosclerotic plaque in both the aortic root and descending aorta.

In Chapter 7 we used bone marrow transplantation technique, reconstituting ApoE<sup>-/-</sup> mice with bone marrow from wildtype mice, to restore apoE function in</sup>macrophages and normalize plasma lipoprotein profiles. This is a novel mouse model with chow diet feeding, providing an alternative model to investigate atherosclerotic plaque regression without robust surgical measures. We performed this regression study in parallel on initial and advanced lesions. We show that the bone marrow transplantation technique leads to successful plaque regression of both initial and advanced lesions. The current mouse model is thus a good model to study atherosclerosis regression in different stages of the disease. However, in initial lesions, a 45% reduction in lesion size was observed with chow diet alone, and even a 71% reduction with LXR agonist treatment, whilst in advanced lesions there was a more limited 23% reduction with chow diet alone, and a 36% reduction with LXR activation. This can be explained by the difference in plague composition and characteristics between initial and advanced lesion. Initial lesions contain primarily cholesterol-filled macrophages covered by a thin cap, while in advanced lesion, SMCs and extracellular matrix products comprise the major structural components of the atherosclerotic plaques, covered by a thick fibrous  $cap^{4'}$ , which make the advanced lesions more difficult to be modulated during the regression process.

Interestingly, our lesion composition analysis showed that the reduction of total lesion size during regression was primarily due to a reduction of the macrophage content in the plaque. However, the fate of the macrophages during lesion

regression is currently still under debate. It has been proposed that regression is not merely a rewinding of progression, but instead involves induction of CCR7 expression, a mediator of leukocyte emigration, leading to the emigration of the lipid-loaded macrophages, followed by the initiation of influx of healthy phagocytes that mobilize necrotic debris and all other components of advanced plaques<sup>48</sup>. Interestingly, Ye et al showed that macrophage infiltration into pre-existing advanced lesions was limited, likely because of the formation of fibrous caps<sup>49</sup>. In contrast. Potteaux et al also showed that regression of atherosclerosis after apoE complementation in ApoE<sup>-/-</sup> mice did not involve migratory egress of macrophages from plaques or induction of CCR7. Instead, marked suppression of monocyte recruitment coupled with a stable rate of apoptosis accounted for loss of plaque macrophages, suggesting that therapies to inhibit monocyte recruitment to plaques may constitute a viable strategy to reduce plaque macrophage burden than attempts to promote migratory egress<sup>50</sup>. Further research is necessary to establish the processes and mechanisms underlying the diminished macrophage content during regression in our experimental mouse model.

In conclusion, the second part of this thesis, focusing on novel mouse models to induce atherosclerotic lesion regression, shows that 1) intact LDL receptor function is crucial to overcome LXR-induced hyperlipidemia as to achieve plaque regression; 2) ApoE<sup>-/-</sup> mice reconstituted with bone marrow from C57BL/6 mice represents a promising mouse model with chow diet feeding to study the regression of atherosclerosis, providing an alternative model to investigate plaque regression without robust surgical measures; and 3) rapidly optimized plasma lipoprotein profiles, combined with LXR agonist treatment, induced favorable gene expression profiles, leading to regression of both initial and more advanced atherosclerotic plaques.

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