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## **Modulation of HDL metabolism : studies in APOE\*3-Leiden.CETP mice**

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### **Citation**

Haan, W. de. (2009, April 16). *Modulation of HDL metabolism : studies in APOE\*3-Leiden.CETP mice*. Retrieved from <https://hdl.handle.net/1887/13730>

Version: Corrected Publisher's Version

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**Note:** To cite this publication please use the final published version (if applicable).

# **Chapter 9**

## **GENERAL DISCUSSION AND FUTURE PERSPECTIVES**

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in the western world, and is mainly caused by atherosclerosis. In the Netherlands, about one third of all deaths are due to CVD. Dyslipidemia (i.e. high plasma (V)LDL-cholesterol (C), high triglycerides and low HDL-C) is a major risk factor for atherosclerosis development and cardiovascular disease. Patients with dyslipidemia are usually treated with cholesterol lowering drugs including statins<sup>1</sup> and fibrates.<sup>2</sup> These drugs lower plasma cholesterol very efficiently (up to 40%),<sup>1,2</sup> however, they prevent only a fraction (about 30%) of cardiovascular events. Therefore new therapeutic strategies to reduce CVD risk more efficiently are necessary. Since HDL is clearly inversely correlated with CVD risk, and has been attributed multiple protective effects in atherosclerosis by its role in reverse cholesterol transport (RCT) and its anti-inflammatory, anti-oxidative and anti-thrombotic properties, HDL-raising therapy is currently considered as a promising strategy to further reduce CVD risk.<sup>3,4</sup>

The research described in this thesis was performed to elucidate the mechanisms underlying the HDL-C raising effects of classic lipid-lowering drugs (fenofibrate, atorvastatin, niacin) as well as an experimental HDL-raising compound (torcetrapib), and to evaluate the HDL-raising potential of novel strategies (PXR agonism, apoCI). The major conclusions and implications of our findings and future perspectives will be discussed here.

### **HDL modulation by classical lipid lowering drugs**

The classical lipid-lowering drugs statins, fibrates and niacin have been shown to modestly raise HDL-C levels in humans up to 10, 15, and 30%, respectively.<sup>5</sup> Interestingly, these drugs failed to show human-like HDL-increasing effects in either normolipidemic or classical hyperlipidemic mice (i.e. LDL receptor-knockout and apoE-knockout mice). Although fenofibrate has been shown to increase HDL in wild-type mice, the raised HDL had an increased particle size, as opposed to the raised HDL in humans. Likewise, although these drugs did evoke human-like lipid-lowering effects in ApoE\*3Leiden transgenic (E3L) mice<sup>6</sup> with respect to dose-dependent decreases of (V)LDL-C and triglycerides, they generally failed to raise HDL-C in E3L mice.<sup>7</sup>

As mice naturally lack the cholesteryl ester transfer protein (CETP), which is an important determinant of HDL metabolism in humans, we reasoned that the HDL-raising effect of the classical lipid-lowering drugs may relate to the presence of CETP. Therefore, we crossbred E3L mice with CETP mice to generate the novel E3L.CETP mouse model.<sup>8</sup> In this thesis we show that E3L.CETP mice indeed respond with an increase in HDL besides a decrease in (V)LDL to both a fibrate (fenofibrate; chapter 2), a statin (atorvastatin; chapter 3) and niacin (chapter 4). This indicates not only that the E3L.CETP mouse is a valuable model to study the effect of HDL modulating drugs on lipid

metabolism, but also that CETP plays an essential role in the HDL increase observed with these drugs.

It is interesting to note that these various classes of drugs have a similar HDL-increasing effect, as dependent on CETP expression, whereas they evoke their lipid-lowering effects through different mechanisms. Statins, fibrates and niacin reduce plasma lipids by primarily by inhibition of the *de novo* hepatic cholesterol synthesis,<sup>9,10</sup> stimulation of triglyceride hydrolysis in plasma,<sup>11-13</sup> and inhibition of lipolysis in adipocytes,<sup>14</sup> respectively. Despite these different primary actions on lipid metabolism, our studies demonstrated that they all reduce the activity and mass of CETP in plasma. In fact, we showed that atorvastatin, fenofibrate and niacin all reduced the hepatic mRNA expression of CETP, which is likely the main causal factor for the reduction in plasma CETP. In line with this hypothesis, increasing the hepatic cholesterol content of CETP transgenic mice by cholesterol feeding increases hepatic CETP expression via LXR-dependent mechanisms as well as plasma CETP mass.<sup>15,16</sup> The three classes of lipid-lowering drugs not only decrease CETP expression, but also decrease (V)LDL levels. Since (V)LDL is an acceptor of HDL-derived cholesteryl esters and, therefore, also a driving force for CETP activity, the decrease in (V)LDL adds to the drug-induced reduction in CETP activity.

Based on our observations, we speculate that lipid-lowering drugs in general will thus all increase HDL-C to a certain extent by reducing plasma CETP activity, as a result of a reduction in both hepatic CETP expression and plasma (V)LDL. In fact, observations in human subjects indeed showed that statins and fibrates both reduce CETP mass and activity.<sup>17-21</sup> The effect of niacin on plasma CETP in humans has not been reported as yet, but niacin will thus probably also reduce CETP mass and activity. Interestingly, hyperlipidemic patients carrying the CETP TaqIB1 polymorphism, who have therefore higher plasma CETP levels than people with the TaqIB2 variant, benefit more from statin treatment with respect to the development of coronary atherosclerosis,<sup>22</sup> suggesting that the reduction of CETP activity is indeed a relevant contributor to the protective effects of lipid-lowering drugs in atherosclerosis.

The apparent robust causal relation between hepatic lipid content and hepatic CETP expression raises the question whether the pathological condition of hepatic steatosis would be a causal factor for reducing HDL-levels by increasing CETP expression. Interestingly, a recent study in obese subjects showed that liver fat indeed inversely correlated with HDL levels.<sup>23</sup> However, since (V)LDL levels are also increased in patients with a fatty liver,<sup>23</sup> more research is needed to confirm the hypothesis that hepatic steatosis results in increased plasma CETP levels thereby decreasing HDL.

**HDL modulation by CETP inhibition**

CETP inhibition has been regarded as a novel promising HDL-C raising strategy to reduce atherosclerosis and cardiovascular disease. Large clinical studies with the CETP inhibitor torcetrapib have been performed to evaluate whether CETP inhibition is able to increase HDL levels and reduce atherosclerosis. Torcetrapib indeed increased HDL-C to a marked extent (+60% at 60 mg/day), but did not reduce atherosclerosis as measured by Intima Media Thickness (IMT) and Intravascular Ultrasound (IVUS)<sup>24-26</sup> and had unwanted effects including increased overall mortality as well as fatal and non fatal cardiovascular events.<sup>27</sup>

There are several possible explanations for the disappointing results of the torcetrapib studies, related to 1) inclusion criteria of the patients, 2) combination therapy with atorvastatin, 3) properties of the newly formed HDL, or 4) compound specific effects.

First, in the torcetrapib studies, patients were included who had undergone cardiac catheterization<sup>26</sup> or have (familial) dyslipidemia.<sup>24,25</sup> These broad inclusion criteria led to the inclusion of a very heterogeneous patient group that may not all benefit from CETP inhibition, as the metabolic context, including baseline CETP activity, HDL-C levels and TG levels, is likely to be an important determinant of the effect of CETP inhibition on atherosclerosis development and CVD risk. The view that the metabolic context is important in the effect of CETP inhibition on atherosclerosis is supported by mouse studies, which in general show that CETP expression is atheroprotective in mice with increased HDL levels while CETP expression is atherogenic in mice with elevated (V)LDL levels. CETP expression in mice with high HDL because of LCAT overexpression or SR-BI deficiency reduces atherosclerosis.<sup>28,29</sup> In line with these mouse data, it has been shown that subjects with highly elevated HDL-C levels which were mainly associated with CETP mutations, have higher prevalence of ischemic ECG changes.<sup>30</sup> CETP inhibition in subjects who have already high HDL may, therefore, not lead to protection against atherosclerosis and CVD. These findings may also suggest that HDL elevation by CETP inhibition is only protective when this normalizes or elevates HDL mildly compared to normolipidemic subjects and CETP inhibitors should not be used to further increase HDL in subjects who have cardiovascular risk factors but normal or elevated HDL levels. In contrast, CETP is a clear atherogenic factor in hyperlipidemic mouse models with impaired (V)LDL clearance, including apoE knockout,<sup>31</sup> LDL receptor knockout<sup>31</sup> and E3L mice.<sup>8</sup> When translated to humans, dyslipidemic subjects with high plasma TG may therefore benefit from CETP inhibition since CETP transfers HDL-CE to (V)LDL that is inefficiently cleared from plasma as indicated by high TG. This suggests that CETP inhibition could be particularly promising in those dyslipidemic patients, who besides elevated (V)LDL also have low HDL and/or high CETP activity.

Further subgroup analyses should be performed in the recent torcetrapib studies to study whether patients with low HDL, high CETP activity and/or high TG at baseline benefit from torcetrapib with respect to atherosclerosis development.

Second, the clinical studies with torcetrapib were all performed in combination with atorvastatin,<sup>24-26</sup> which by itself reduces CETP activity in plasma.<sup>17-20,32,33</sup>

By investigating the effect of torcetrapib on atherosclerosis development in E3L.CETP mice, either in combination with atorvastatin or alone (chapter 5), we showed that torcetrapib *per se* did in fact reduce atherosclerosis while torcetrapib did not reduce atherosclerosis in mice that were also treated with atorvastatin, indicating that combination treatment with atorvastatin and other lipid lowering drugs may attenuate or mask the effect of torcetrapib on atherosclerosis.

Third, it is not clear whether the increased HDL as induced by CETP inhibition by torcetrapib is atheroprotective or not. HDL is thought to be protective in atherosclerosis via mediating RCT and by its proposed anti-inflammatory, anti-oxidative and anti-thrombotic properties. Torcetrapib alters HDL by increasing its size, by the formation of a torcetrapib/CETP complex that associates with HDL, and torcetrapib may alter the protein composition of HDL which may alter HDL functionality. In addition, torcetrapib may reduce HDL-CE clearance as it has been described that in humans, the majority of HDL-CE reaches the liver via (V)LDL after CETP-mediated transfer of HDL-CE from HDL to (V)LDL, and this pathway is blocked by torcetrapib.<sup>34,35</sup>

Fourth, torcetrapib may have had compound-specific adverse side effects with respect to increased mortality and increased cardiovascular events, and compound-specific side effects may explain why torcetrapib did not add to the atherosclerosis-reducing effect of atorvastatin. Torcetrapib has been found to increase blood pressure by approx. 5 mm Hg,<sup>24-26,36</sup> which is not observed with novel CETP inhibitors including anacetrapib and JTT-705. This mild increase in blood pressure is unlikely to completely have counteracted potential protective effects of the HDL increase and is unlikely to have caused increased mortality, but it may be indicative for other underlying adverse effects. Analysis of plasma samples from patients treated with torcetrapib showed an increase in sodium, bicarbonate and aldosterone levels.<sup>27</sup> Torcetrapib also increased plasma aldosterone in E3L.CETP mice (chapter 5). The raise in aldosterone may not only explain the increase in blood pressure, but animal studies have also shown that aldosterone causally increases atherosclerosis, inflammation and oxidative stress,<sup>37-40</sup> indicating that the increase in aldosterone may have counteracted potentially atheroprotective effects of the torcetrapib-induced increase in HDL. We have demonstrated that torcetrapib increases the macrophage to collagen ratio within atherosclerotic plaques in E3L.CETP mice (chapter 5). Whereas plaques of mice do not easily rupture spontaneously, such a phenotype is considered more prone to spontaneous rupture in humans. Extrapolation of our data to humans may thus suggest that the increase in cardiovascular events and

death may have been caused, at least partly, by increased incidence of plaque rupture. As aldosterone is associated with more inflammation and increased activity of matrix metalloproteinases (MMP),<sup>39,41</sup> which cause breakdown of collagen, it can be reasoned that the increased aldosterone levels may have contributed to the plaque phenotype. However, this hypothesis should be underscored by experimental studies. The effects of torcetrapib on aldosterone and blood pressure are compound-specific and independent of CETP, as torcetrapib induced these effects in both humans, CETP transgenic mice and non-transgenic mice.<sup>42</sup> In contrast, anacetrapib does not increase blood pressure and aldosterone levels.<sup>42,43</sup> The effect of novel CETP inhibitors on plaque composition and cardiovascular events is thus eagerly awaited.

In addition to chemical CETP inhibitors, endogenous CETP inhibitors have also been described. Whereas the lipid transfer inhibitor protein (LTI) as present on LDL inhibits CETP activity,<sup>44</sup> apoCI is the major endogenous CETP inhibitor present on HDL.<sup>45</sup> being an endogenous protein, apoCI may be a lead for novel safe CETP inhibitors. However, apoCI is also an inhibitor of LPL.<sup>46</sup> Because of apoCI-induced LPL inhibition, human apoCI overexpression in CETP transgenic mice not only reduces specific CETP activity, but also largely increases VLDL levels. The increase in VLDL levels consequently increases hepatic CETP gene expression, which precludes an increase in HDL-C resulting from CETP inhibition only.<sup>47</sup>

To find an apoCI derived CETP inhibitor without LPL inhibitory properties, we performed structure-function analysis using an array of apoCI derived peptides. ApoCI<sub>32-57</sub> was the most efficient CETP inhibitory peptide tested, and this peptide had only minimal effects on LPL. Therefore we expect that this peptide should be able to increase HDL without inducing hyperlipidemia. Pilot experiments showed that intravenous injections with apoCI and apoCI<sub>32-57</sub> were unable to modulate lipid levels *in vivo* in CETP transgenic mice (unpublished data), which may relate to dosing and/or adverse pharmacokinetics of the peptides. Short-term elevation of plasma levels of apoCI and apoCI<sub>32-57</sub>, e.g. by using recombinant adenoviruses that induce a relatively high hepatic protein expression of apoCI and apoCI<sub>32-57</sub> in CETP expressing mice could therefore be useful as a tool to show the potential of apoCI<sub>32-57</sub> to increase HDL levels without affecting plasma TG. If so, apoCI<sub>32-57</sub> mimicking agents could be developed that can be used orally.

In addition to CETP, apoCI also affects other HDL modifying enzymes, including LCAT<sup>48,49</sup> HL<sup>50,51</sup> and SR-BI (chapter 7). These combined actions increase HDL in naturally CETP-deficient wild-type mice (chapter 7), but functionality of the increased HDL is unknown. It is also unknown how apoCI<sub>32-57</sub> would act on these various proteins involved in HDL metabolism. Therefore, additional *in vitro* and *in vivo* experiments in wild-type mice should be performed to show whether potential HDL-increasing effects of apoCI<sub>32-57</sub>

are CETP dependent. In addition, studies in E3L.CETP mice will be useful to reveal whether apoCI<sub>32-57</sub> will reduce atherosclerosis. It would be interesting to study the effect of apoCI<sub>32-57</sub> on the plaque phenotype as well as to evaluate whether the effect of torcetrapib on plaque composition was indeed compound specific, especially because apoCI has a different mechanism to inhibit CETP as compared to torcetrapib, i.e. reduction of affinity of CETP with HDL<sup>52</sup> versus formation of an inactive HDL/CETP complex.<sup>53</sup>

### **Novel strategies to reduce cardiovascular disease**

Cholesterol lowering is a proven effective strategy to reduce cardiovascular disease. Therefore a large number of new drugs to reduce plasma lipid levels is under development, including microsomal triglyceride transfer protein (MTP) inhibitors, squalene synthase inhibitors, and apoB expression inhibitors that are all aimed at reducing lipid production by the liver.<sup>54-58</sup> However, as statins already efficiently reduce plasma (V)LDL-C levels without severe side effects, and additional safe (V)LDL-C lowering agents to treat patients that do not respond well to statins are available (e.g. cholesterol binding resins) it will be difficult to develop novel lipid lowering drugs that lead to more clinical benefit with respect to protection against cardiovascular disease.

After the disappointing results from the torcetrapib studies, it is difficult to predict whether novel HDL raising agents will prevent CVD in the future. Despite that there is no direct evidence for the protective effect of HDL in atherosclerosis, HDL is thought to play a role in RCT, and is thought to be anti-inflammatory, anti-oxidative and anti-thrombotic. HDL raising may be achieved by 1) reducing HDL clearance, 2) increasing HDL maturation and modification of HDL metabolism or 3) enhancing HDL production.

In mice, clearance of HDL-C is almost exclusively mediated via SR-BI. In humans, Cla-1 (i.e. the human orthologue of SR-BI) is thought to play a less important role, since HDL-CE is mainly cleared from plasma after CETP-mediated transfer to (V)LDL.<sup>34</sup> Albeit that raising HDL by reducing its clearance may increase the anti-inflammatory and anti-oxidative properties of HDL, the role of HDL in RCT (i.e. transfer of cholesterol to the liver, followed by excretion via the feces) is possibly the most important protective function of HDL. Together with the fact that SR-BI-deficiency in mice aggravates atherosclerosis, reducing HDL clearance may probably not be the most valid HDL-raising strategy.

LCAT plays an important role in the maturation of HDL as this enzyme esterifies HDL-associated cholesterol into CE. Mutations in LCAT which lower LCAT activity reduce HDL-C levels and mildly enhance atherosclerosis development (as measured by IMT).<sup>59</sup> However, since overexpression of LCAT in mice increases atherosclerosis, LCAT-targeted interventions to raise HDL should be pursued with care. As niacin increases HDL via CETP reduction



(chapter 4) niacin can also be considered as a compound that increases HDL via HDL modulation. Niacin is however not well tolerated because it induces severe flushing. A recent study shows that addition of the prostaglandin D<sub>2</sub> receptor 1 blocker laropiprant reduces niacin mediated flushing which may increase niacin use.<sup>60</sup> In addition novel compounds targeting the niacin receptor GPR109A are under development.<sup>61</sup> However, it is uncertain whether the protective effects of niacin on IMT progression and mortality are mediated via its HDL raising or via its (V)LDL reducing properties<sup>62,63</sup>

HDL production is presumably mainly initiated by the synthesis and secretion of apoAI by the liver and intestine, and lipidation of apoAI in plasma via ABCA1. Indeed, apoAI-deficiency and ABCA1-deficiency in mice largely decrease HDL-C. Interestingly, humans carrying mutations in ABCA1 and apoAI<sup>59</sup> have a more severe increase in atherosclerosis (as measured by IMT) as compared to carriers of CETP or LCAT mutations, suggesting that enhancing HDL production would be the most promising strategy in the prevention of atherosclerosis and CVD. Upregulation of ABCA1 in macrophages can be achieved with compounds like LXR agonists.<sup>64</sup> However, these compounds will also induce the expression of lipogenic genes in the liver which counteract potential protective effects of ABCA1 upregulation in atherosclerosis. In addition, LXR activation will result in upregulation of multiple proteins involved in cholesterol efflux, including apoE, which hampers evaluation of the effect of upregulation of ABCA1 only. Therefore, compounds that specifically upregulate ABCA1 expression in macrophages, preferably in the atherosclerotic vessel wall, should be developed. Increase of apoAI can be achieved via administration of apoAI or apoAI mimicking compounds, or via upregulation of apoAI expression. ApoAI, infused either as a lipid-free protein or contained in recombinant HDL, increases HDL levels. Clinical studies show that short-term apoAI infusion lead to a quick reduction of atheroma volumes in patients with acute coronary syndrome.<sup>65</sup> However, a drawback of apoAI infusion is that treatment is invasive and very expensive, and that long term effects are still unknown.<sup>66</sup> Alternatively, apoAI mimicking agents have been developed which can be given orally.<sup>67</sup> Agents that increase apoAI production would also be promising to increase endogenous HDL production. It has been suggested from studies in wild-type mice that PXR agonism would increase apoAI production and raise HDL levels.<sup>68</sup> However, we observed in our more human-like E3L.CETP mice that PXR agonists reduced HDL of all sizes concomitant with a reduction (rather than an increase) in apoAI expression (chapter 6). This study thus indicates that PXR agonism is likely also unable to raise apoAI and HDL in humans. Recently, rvx-208 has been developed to induce apoAI expression and raise HDL levels, as shown in mice and non-human primates. Provided that this compound is specific for apoAI, it would be promising new lead in the ongoing search for HDL-raising strategies to prevent CVD.<sup>69</sup>

## Concluding remarks

Besides a large number of new lipid-lowering agents, drugs that are aimed to specifically raise HDL are currently under development. The therapeutic value of such HDL-raising therapies, however, is still unclear, especially since the first HDL-raising strategy by torcetrapib failed in large human trials. The question whether raising HDL will add to the atheroprotective effect of lipid-lowering therapy is thus still unanswered. Albeit that high HDL-C is clearly associated with reduced CVD risk, high HDL-C is also associated with low (V)LDL-C and TG, a balance that is probably dictated by bidirectional transfer of neutral lipids by CETP. Therefore, it is difficult to predict the importance of HDL independent of other risk factors, and virtually no direct evidence is currently available to show that HDL *per se* is protective in atherosclerosis development. Therefore, other therapeutic strategies such as inhibition of inflammation, which besides dyslipidemia is also a driving force for atherosclerosis, should not be overlooked.

The torcetrapib trials taught us that care should be taken in selecting appropriate subjects in human studies that are expected to benefit most from a novel experimental approach to reduce CVD. Future studies will show which of these new strategies will eventually be used in the future therapy of those patients that are prone to develop CVD. Moreover, our studies indicate the importance of testing effects of experimental drugs on lipid metabolism, atherosclerosis and plaque composition in appropriate animal models. We expect that our newly developed E3L.CETP mouse model with a human-like lipoprotein metabolism will largely contribute to the development of such compounds by reliably predicting human responses to experimental drugs and revealing underlying mechanisms.

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