Hyperlipidemia, inflammation and atherosclerosis : roles of apolipoprotein C1 and cholesteryl ester transfer protein

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CHAPTER 7

General Discussion and Future Perspectives

General discussion and future perspectives

Cardiovascular disease (CVD) is the first cause of death in the Western world and its prevalence is increasing in Eastern Europe and developing countries. The main cause of CVD is atherosclerosis. Since hyperlipidemia and inflammation are independent risk factors for atherosclerosis, many genes affecting these disorders are suggested to affect atherosclerosis development.[1-3] The role of apolipoprotein CI (apoCI) in atherosclerosis is not completely understood, and the role of cholesteryl ester transfer protein (CETP) in atherosclerosis is still undergoing debate. The research in this thesis was designed to gain more insights into the role of these proteins in lipoprotein metabolism, inflammation, and atherosclerosis. The major conclusions and implications of our findings, and the future perspectives will be discussed here.

Role of apoCI in VLDL metabolism

It has been shown that human *APOC1* overexpression in wild-type mice consistently leads to increased very-low-density-lipoprotein (VLDL)-triglycerides (TG) and VLDL-cholesterol.[4-8] Initially, these effects were mainly explained by inhibition of apoE-mediated uptake of VLDL via the low density lipoprotein receptor (LDLr) and LDLr related protein (LRP).[6;7;9-11] However, in *apoe*$^{-/-}$ mice, VLDL-TG levels are not affected.[12-14] Recently we found that human apoCI overexpression causes hypertriglyceridemia even in the absence of apoE,[4;5] and we showed that the mechanism underlying this observation was apoCI-induced inhibition of lipoprotein lipase (LPL).[4]
In this thesis, it was shown that physiological levels of endogenous murine apoCI increase VLDL-TG and VLDL-cholesterol in *apoe*$^{-/-}$ mice. Similarly as for human apoCI overexpression, this can be explained by apoCI-induced LPL inhibition, yet also by apoCI-induced increased VLDL particle production.[15] In wild-type mice, endogenous apoCI expression shows only a tendency to increase VLDL-TG,[16-19] which might depend on the same mechanisms. The effects of endogenous apoCI on VLDL metabolism are thus similar to the effects of human apoCI overexpression, regarding LPL inhibition yet not regarding VLDL particle production, which was unaffected in *APOC1* transgenic mice.[4] Endogenous apoCI may be necessary for VLDL particle production, yet overexpression of apoCI can not further enhance the effect.

Hence, increased VLDL-TG levels can be explained by LPL inhibition in *apoe*$^{-/-}$ mice expressing endogenous apoCI,[15] and in wild-type and *apoe*$^{-/-}$ mice overexpressing human apoCI.[4] How does this effect relate to other apolipoproteins that affect LPL activity, including apoCII, apoE, and apoCIII? ApoCII is the cofactor for LPL and needs to be present for the enzyme to be active.[20;21] Physiological apoE expression and human apoE overexpression inhibit LPL.[22-25] Although, if we compare wild-type mice to *apoe*$^{-/-}$ mice, apoE appeared to be even more important in mediating the remnant clearance via uptake by the LDLr, LRP, and HSPG on the liver,[26-31] as evident from severe hypercholesterolemia in *apoe*$^{-/-}$ mice,[12-14] rather than a function in VLDL-TG lipolysis. Finally, apoCIII has been shown to inhibit LPL,[32;33] and to date, is considered the most potent LPL inhibitor. As for apoCI, the effect of apoCIII on hypertriglyceridemia in wild-type mice has also been initially explained by inhibition of apoE-mediated uptake of TG-remnant lipoproteins by the liver,[34] yet later studies in *apoe*$^{-/-}$*APOC3* mice showed that the main role of apoCIII is LPL inhibition.[35] These results indicate that apoCI and apoCIII have similar roles in VLDL clearance. However, apoCIII is more effective regarding LPL inhibition than apoCI, as shown by the following results: *1*) apoCIII is twice as effective as apoCI in inhibiting LPL activity *in vitro*,[4] *2*) human apoCIII transgenic (*APOC3*) mice show 2-fold higher VLDL-TG levels than *APOC1*$^{+/0}$ mice, whereas the plasma levels of both apolipoproteins are similar (40-50 mg/dl),[4;36] *3*) the presence of endogenous apoCIII on a wild-type background increases TG levels by 180%,[37] whereas endogenous apoCI only shows a tendency to increase plasma TG by 33%,[16-19] and *4*) the presence of apoCIII shows a more pronounced TG increase in *apoe*$^{-/-}$ mice (570%),[37] than the presence of apoCI (137%).[15]

Taken together, while apoCI, apoCII, apoCIII, and apoE all affect VLDL metabolism, their individual contribution to VLDL production and clearance are clearly different. Since the major role of apoE in VLDL metabolism is mediating the uptake of VLDL particles, and apoCI inhibits the apoE-mediated VLDL uptake,[6;7;9-11] apoCI has an opposite role to apoE. In addition, apoCI has an opposite role to apoCII with respect to modulation of LPL activity. ApoCI seems to be most similar to that of apoCIII with respect to their roles in VLDL clearance. ApoCIII inhibits the apoE-mediated uptake of remnants and inhibits LPL, similar to apoCI. Although apoCI is less effective than apoCIII in the inhibition of LPL, we have shown that expression of apoCI

inhibits LPL, thereby contributing to increased VLDL-TG levels in mice.[4;15] It is intriguing to speculate about the mechanism behind apoCI-induced LPL inhibition. It has been shown that overexpression of human apoCI inhibits LPL independently of the endogenous expression of apoE, apoCIII, and the VLDLr, the latter regarded as the chaperone protein for LPL.[4;38] As we now found that also physiological expression levels of endogenous murine apoCI are able to inhibit LPL in *apoe*$^{-/-}$ mice, LPL inhibition as induced by endogenous murine apoCI is thus also independent of apoE. Furthermore, endogenous apoCI inhibits LPL independent of active LPL levels in plasma and hepatic expression levels of *apoc2* and *apoc3*, as all of these levels were not affected by endogenous apoCI expression.[15] In addition, VLDL from mice expressing endogenous apoCI was less reactive to soluble LPL *in vitro*, indicating that apoCI does not necessarily act by preventing the interaction of VLDL with heparan sulfate proteoglycans (HSPG) that connect LPL with the endothelium.[15] In fact, we have shown by *in vitro* LPL activity assays that apoCI can directly inhibit LPL activity.[4] Since we used an excess of FA-free bovine serum albumin (BSA) that rapidly sequesters FA generated by LPL-induced TG hydrolysis, potential product-inhibition of LPL by generated FA was excluded. As, in this assay, except for apoCII, no other apolipoproteins were added, we suggest two mechanisms underlying the apoCI-mediated LPL inhibition: *1*) apoCI directly interacts with apoCII, the cofactor for LPL, thereby displacing apoCII from the particle or masking its presence; and *2*) apoCI directly interacts with LPL, thereby masking the catalytic domain of LPL or reducing the affinity of LPL for VLDL. The first mechanism seems to account for the LPL-inhibiting properties of apoCIII and apoE, as both apoCIII[32] and apoE3[39] have been shown to displace apoCII, the cofactor for LPL, from particles. The positive arginine residues of apoE play an important role in LPL inhibition, since elimination of their charges by modification with cyclohexadione completely abolished its inhibitory effects.[24] As apoCI is also rich in positively charged amino acids, *i.e*. arginine and lysine residues,[40-43] the mechanism underlying apoCI-induced LPL inhibition could parallel the mechanism of apoE. In addition, apoCIII has been postulated to inhibit LPL activity independent of binding to VLDL.[32] Likewise, apoCI could also directly interact with LPL, thereby preventing it to associate with its substrate or mask its catalytic domain. The relative importance of both mechanisms should be further investigated.

Interestingly, we showed in this thesis that apoCI binds free fatty acids. In *APOC1*$^{+/0}$ and *APOC1*$^{+/+}$ mice, FFA levels on VLDL are increased concomitantly with apoCI. The increased FFA on VLDL appeared to be the consequence of apoCI-mediated bridging of FFA to VLDL, as we showed *in vitro*. This property of apoCI may add to its LPL-inhibiting activity, as it is well known that FFA generated by LPL can modulate LPL activity by product inhibition. Since apoCI apparently binds FFA, thereby retaining FFA on the surface of VLDL, apoCI may thus enhance production inhibition of LPL. This might, at least partly, explain the TG-raising effect of human apoCI overexpression in *APOC1*$^{+/0}$ and *APOC1*$^{+/+}$ mice. However, such a mechanism does probably not contribute to the LPL inhibition by endogenous apoCI expression on the *apoe*$^{-/-}$ background, as FFA levels on VLDL, and also in whole plasma, were not affected upon endogenous apoCI expression.[15]
An interesting observation was that the binding of apoCI to FFA depended on the lysine (*K*) residues of the *KVKEKLK* motif in apoCI. Presumably, electrostatic interactions between the negatively charged carboxyl group of FFA and the positively charged lysine residues of apoCI contribute to apoCI-FFA binding. Compared to all other known apolipoproteins, apoCI exhibits the highest isoelectric point and the highest lysine content (16 mol%),[40;41;43] which may indicate that only apoCI can bind FFA, and other apolipoproteins do not exert this property. Indeed, FFA-binding properties of other apolipoproteins have not been reported before.

ApoCI overexpression has been shown to protect mice against diet-induced obesity and obesity on the *ob/ob* background, as shown in mice overexpressing human apoCI homozygously (*APOC1*$^{+/+}$ mice).[44] The findings presented in this thesis that apoCI inhibits LPL and binds FFA, thereby preventing the cellular uptake of FFA, can both form mechanistic explanations underlying this observation. Reduced LPL activity has been shown to consistently protect against obesity development in several mouse models. For example, adipose tissue-specific LPL-deficient mice show reduced obesity development on the *ob/ob* background.[45] In addition, mice lacking the VLDLr, the chaperone protein required for efficient translocation of LPL to the endothelium, are protected against obesity on the *ob/ob* background,[46] which may be the result of reduced LPL activity, as demonstrated in *vldlr*$^{-/-}$ mice.[47] Furthermore, expression of endogenous apoCIII, probably the most effective LPL inhibitor,

leads to reduced diet-induced obesity.[48] Thus, protection against both diet-induced obesity and obesity on the *ob/ob* background in *APOC1*$^{+/+}$ mice[44] may be the direct consequence of apoCI-induced LPL inhibition.
In addition, we showed that apoCI binds to FFA, a mechanism by which we explained our finding that apoCI reduces cellular FFA association and subsequent intracellular FFA esterification in cells. In line with this hypothesis, apoCI may also prevent the uptake of FFA by adipocytes and thus contribute to prevention of obesity. Indeed, reduced FFA uptake by adipose tissue was initially thought to underlie the reduced obesity in *APOC1*$^{+/+}$*.ob/ob* mice.[44] Similarly, reduced FFA uptake by the skin may be the explanation for the dry and scaly skin of *APOC1*$^{+/+}$ mice.[49] FFA esterification into wax esters is necessary for the production of sebum that is suggested to prevent the skin from water loss.[49] The idea that FFA esterification is necessary for both sebum production and the storage of TG in adipose tissue is further supported by the concomitant finding of cutaneous abnormalities and protection against obesity in mouse models that lack genes affecting the intracellular esterification or synthesis of FA: the acyl CoA:diacylglycerol acyltransferase 1 deficient (*dgat1*$^{-/-}$) mouse[50;51] and the stearoyl-CoA desaturase 1 deficient (*scd1*$^{-/-}$) mouse,[52-54] respectively.

The role of apoCI in atherosclerosis

We questioned how the effects of apoCI on VLDL-TG metabolism relate to atherosclerosis development. It has been shown that reduced systemic LPL expression (thus reduced LPL activity in the capillary beds in plasma) increases atherosclerosis in *ldlr*$^{-/-}$ and *apoe*$^{-/-}$ mice, as probably caused by enhanced hyperlipidemia.[55;56] We found that systemic physiological apoCI expression increased atherosclerosis, probably as a consequence of enhanced levels of VLDL-TG and VLDL-cholesterol due to apoCI-induced systemic LPL inhibition.[57] Next to an effect of systemic apoCI expression on plasma lipid levels and atherosclerosis, apoCI has been suggested to affect lipid homeostasis in cells locally. ApoCI has been shown to be capable of inducing cholesterol efflux from human epithelial cells (Hela) cells, via the activity of the ATP binding cassette A1 (ABCA1) transporter.[58] This study actually showed that next to the established cholesterol acceptors apoAI and apoE,[59] many other apolipoproteins were capable of inducing ABCA1-mediated cho-

lesterol efflux,[58] including apoAII, apoAIV, apoCII, and apoCIII.[58] It appeared that the presence of an amphipathic helix within apolipoproteins is required to induce ABCA1-mediated cholesterol efflux rather than the amino acid sequence of the apolipoprotein *per se*.[58] However, apoAI and apoE expression protect against atherosclerosis development related to induced cholesterol efflux from macrophages instead of epithelial cells,[60;61] and the effect of apoCI on cholesterol efflux from macrophages has not been investigated before. Several lines of evidence suggested a role for apoCI in macrophage lipid homeostasis. ApoCI is upregulated 85-fold during monocyte to macrophage differentiation[62] and 25-fold upon treatment with an agonist for the lipid sensor the liver X receptor (LXR).[63] Therefore, in this thesis we investigated whether apoCI induces cholesterol efflux from macrophages. Indeed apoCI was a potent cholesterol acceptor, with similar efficacy as compared to apoE and apoAI.[57] In addition, apoCI-induced cholesterol efflux from macrophages was mediated by ABCA1,[57] which has also been shown in Hela cells.[58] Although apoCI is thus a potent cholesterol acceptor, apoCI expression in the vessel wall did not affect atherosclerosis development as we demonstrated by investigating the effect of apoCI in macrophages locally, using *apoe$^{-/-}$apoc1$^{+/+}$* mice transplanted with bone marrow from *apoe$^{-/-}$apoc1$^{-/-}$* versus *apoe$^{-/-}$apoc1$^{+/+}$* mice.[57] Presumably, the concentration of apoCI in the atherosclerotic plaque is not high enough to induce as much cholesterol efflux as to reduce atherosclerosis development. The finding that systemic apoCI expression also resulted in increased rather than decreased atherosclerosis, indicates that the overall effect of plasma apoCI is rather to increase lipid influx into the atherosclerotic plaque as a consequence of inducing hyperlipidemia than to increase lipid efflux by its cholesterol-accepting properties.

To extend our observations on the effect of apoCI on VLDL-TG metabolism and atherosclerosis to the human situation, it is of interest to note that in human carriers of the *HpaI* polymorphism that has been shown to result in increased expression of the apoCI gene, increased apoCI levels have been associated with increased TG levels.[64] However, as apoE levels were also affected, and the increased apoCI expression was in linkage disequilibrium at least with the expression of apoE2 and apoE4, yet not apoE3, apoCI cannot be identified as a causal factor for the increased TG levels.[64]

With respect to the effect of apoCI on coronary artery disease (CAD), it has been reported that the apoCI content of TG-rich lipoproteins independently predicts early atherosclerosis in healthy middle-aged men.[65] This result is in agreement with our findings that apoCI on VLDL accelerates atherosclerosis development in mice.[57] Further research is warranted in order to explore how apoCI expression in humans relates to CAD.

Role of apoCI in HDL metabolism

Apolipoproteins that induce ABCA1 mediated cholesterol efflux from macrophages have also been shown to be important in inducing ABCA1 mediated cholesterol efflux from other cell types, thereby contributing to HDL formation. For example apoAI-induced ABCA1-mediated cholesterol efflux from macrophages protects against atherosclerosis development,[60] and the apoAI-induced ABCA1-mediated cholesterol efflux from the liver[66] and the intestine[67] contribute to the formation of nascent HDL. ApoCI could also play a role in this process, since the majority of apoCI circulates on HDL,[68] and apoCI is capable of inducing ABCA1 mediated efflux from other cell types.[57;58] A potential role in HDL formation is corroborated by the finding that *apoc1*$^{-/-}$ mice show reduced HDL cholesterol (~50%),[17] and wild-type mice injected with a human apoCI-expressing recombinant adenovirus show increased HDL-cholesterol (C.C. van der Hoogt *et al*., unpublished observations). In addition, apoCI has been shown to modulate several enzymes involved in HDL metabolism that could explain its effects in increasing HDL-cholesterol in wild-type mice. ApoCI activates lecithin:cholesterol acyl transferase (LCAT), with an efficacy of 10-45% of the main LCAT activator apoAI, depending on the lipid substrate.[69] Also apoE2, apoE3, and apoAIV are modest LCAT activators, albeit weaker compared to apoAI.[70;71] As for their cholesterol-efflux inducing properties, the LCAT stimulating effects of these apolipoproteins were shown to be rather due to the amphipathic helices present within these apolipoproteins than to their specific amino acid sequence.[71] Interestingly, the size of the HDL that was formed in wild-type mice injected with the apoCI adenovirus was very similar to that of large HDL_1 as observed in *LCAT* transgenic mice, indicating that the increased HDL size may be the consequence of LCAT activation *in vivo*.[72]

Next to potentially enhancing HDL generation and HDL-cholesterol esterification, apoCI may increase HDL levels by affecting HDL clearance. Hepatic lipase is involved in HDL remodelling and subequent clearance of HDL, as demonstrated by increased HDL-cholesterol levels in *hl*$^{-/-}$ mice.[73] *In vitro* studies have demonstrated that apoCI inhibits hepatic lipase (HL) activity.[5;74] However, since apoCIII has been shown to inhibit HL *in vivo*, while HDL levels are not affected,[74] it is unlikely that potential HL-inhibiting properties of apoCI will substantially contribute to increased HDL levels. Also *scavenger receptor b1*-deficient (*srb1*$^{-/-}$) mice express large HDL_1,[75] indicating that apoCI may inhibit the selective HDL-CE uptake via SR-BI. The uptake of HDL-CE by SR-BI is mediated in part by apoE.[76] The findings that apoCI masks the presence or displaces apoE from remnant particles, thereby reducing the uptake of remnants by the liver via the LDLr and the LRP,[6;7;9-11] may corroborate a role for apoCI in masking or displacing apoE also from HDL, and reducing HDL-CE uptake by the liver via SR-BI.
With regard to the current interest in HDL-increasing therapies as a novel target to treat CVD, it will be interesting to elucidate by which of these factors apoCI influences HDL production and clearance, thereby increasing HDL levels, and whether this insight can lead to apoCI-based therapeutic strategies to reduce cardiovascular risk.

Role of apoCI in CETP modulation: implications for VLDL and HDL metabolism

The cholesteryl ester transfer protein (CETP) mediates the exchange of CE and TG between apoB-containing lipoproteins (*i.e.* chylomicrons, VLDL, and LDL) and HDL,[77] and connects as such the metabolism of HDL with that of apoB-containing lipoproteins. CETP is expressed in many species, including humans, yet not in rodents such as mice.[78] Interestingly, apoCI is a potent endogenous inhibitor of CETP activity, and even appeared to be the only apolipoprotein on HDL capable of inhibiting CETP.[40] This role of apoCI may be contributed to its high content of positively charged amino acids, as it was shown that increasing the positive charge of HDL by binding of apoCI was associated with reduced affinity of CETP for HDL.[79] Such a finding is corroborated by preliminary results showing that the apoCI mutant, in which the positive lysine (*K*) residues in the *KVKEKLK* motif of apoCI have been

replaced by neutral alanine (*A*) residues, is much less effective in CETP inhibition (W. de Haan *et al*., unpublished observations).

Studies in *apoc1$^{-/-}$.CETP* versus *apoc1$^{+/+}$.CETP* mice showed that apoCI can also reduce CETP activity *in vivo*. ApoCI expression decreased the endogenous CETP activity, and resulted in increased HDL-cholesterol and decreased VLDL-TG.[16] In this study, the effect of apoCI on TG levels was caused by the effect of HDL-bound apoCI on CETP activity, since endogenous apoCI expression in wild-type mice without CETP did not substantially affect VLDL-TG metabolism by inhibition of LPL.[16-19] Although human apoCI overexpression also reduced the specific CETP activity in *APOC1$^{+/0}$* mice, decreased HDL-CE and increased VLDL-TG levels were observed.[80] This apparent paradoxical observation can be explained by the concurrent lipid-raising effect of apoCI overexpression in these mice.[4] Increased VLDL-CE levels in the circulation of *APOC1$^{+/0}$.CETP* mice probably led to LXR activation in the liver, thereby inducing its responsive genes, including CETP.[80] As a result, CETP transcription was upregulated to such a large extent, that an inhibiting effect of apoCI on CETP activity was overruled by a severely increased CETP transcription.[80] Therefore, in *APOC1$^{+/0}$.CETP* mice the overall effect of apoCI on plasma lipid levels is primarily determined by the inhibiting effect of apoCI on LPL rather than by the inhibiting effect on CETP.[80] Also in *apoe$^{-/-}$apoc1$^{-/-}$* and *apoe$^{-/-}$apoc1$^{+/+}$* mice, physiological levels of endogenous murine apoCI increase plasma VLDL levels.[15] It would be interesting to examine whether the atherogenic effect of the apoCI-induced increase in VLDL would be counteracted by a potentially concomitant increase in HDL resulting from CETP inhibition.

From our data on the role of apoCI in atherosclerosis in mice and the association of apoCI with CAD in humans, it seems that apoCI has an overall proatherogenic role in humans and mice by increasing hyperlipidemia that mainly results from LPL inhibition. However, in view of our observation that CETP increased the diet-induced atherosclerosis in the humanized *APOE*3-Leiden* mouse model,[81] and the interest in CETP inhibitors as a therapeutic target, it is tempting to speculate how we can modulate the sequence of apoCI to make it an effective CETP inhibitor while not affecting LPL activity. The preliminary findings that the whole sequence of apoCI is necessary for LPL inhibition, and that a fragment containing the C-terminal helix of apoCI (amino acid residues 32-57) can inhibit CETP without inhibiting LPL (W. de

Haan *et al*., unpublished observations), make it interesting to test the effect of this apoCI fragment on the cholesterol distribution over lipoproteins and atherosclerosis development in *CETP.APOE*3-Leiden* mice.

The role of apoCI in inflammation

In addition to its roles in lipoprotein metabolism, apoCI plays an important role in inflammation. ApoCI binds to lipopolysaccharide (LPS) at low concentrations (10-100 ng/ml), thereby inducing the LPS-mediated response to macrophages *in vitro* and in mice *in vivo*.[82] This was, as for many of its properties in lipoprotein metabolism, dependent on its positively charged lysine (*K*) residues in the *KVKEKLK* motif, since the apoCI mutant, containing neutral alanine (*A*) residues instead, was much less effective.[82] Since the lipid A moiety of LPS exhibits two negatively charged phosphate groups, binding of apoCI presumably involves electrostatic interaction between the lysine residues within apoCI and the negatively charged phosphate groups within LPS. ApoCI appeared to be protective in sepsis, by effectuating an efficient inflammatory response.[82] Related to this finding, we now show that upon chronic LPS treatment of *apoe*$^{-/-}$*apoc1*$^{-/-}$ versus *apoe*$^{-/-}$*apoc1*$^{+/+}$ mice, apoCI enhances the LPS-induced expression of inflammatory markers and is crucial for the LPS-induced atherosclerosis development. It is thus likely that apoCI is also pro-atherogenic in the context of chronic infection as induced by Gram-negative bacteria.
This LPS-binding property is not unique for apoCI. ApoE has also been shown to bind LPS.[83] However, whereas apoCI increases the LPS-induced inflammatory response, binding of LPS to apoE leads to suppression of the LPS response.[83] Thus, apoCI and apoE do not only have opposite effects on the clearance of TG-rich lipoprotein remnants, yet also have opposite effects on LPS-induced inflammation. With respect to their effects on lipid metabolism, apoCI is pro-atherogenic, whereas apoE is atheroprotective.[12-14;61] An effect of apoE on LPS-induced atherosclerosis has not been determined yet, but apoE expression is presumably atheroprotective in this context. In addition, human apoAIV overexpression decreased the LPS-induced atherosclerosis in *apoe*$^{-/-}$ mice, related to the presence of less inflammatory cytokines.[84] Since infections of humans with Gram-negative bacteria such as *Chlamydiae pneumoniae* have been associated with increased CVD,[85-87] it will be

interesting to investigate whether apoCI contributes to this effect in humans as well.
The induction of the inflammatory response by apoCI might be an additional explanation for the cutaneous abnormalities of *APOC1*$^{+/+}$ mice. As a result of the lack of sebum production, the hair canals in the skin are more susceptible to the entry of bacteria,[49] and apoCI expression may further locally enhance the resulting inflammation. Furthermore, the fact that apoCI increases inflammation may also have implications for the development of inflammation related insulin resistance and type II diabetes. The mechanism underlying the apoCI-induced increased LPS response has not been clarified yet. As the Toll-like receptor 4 (TLR4)/MD2 complex mainly mediates the LPS response[88] and plays a role in atherosclerosis,[89] the TLR4/MD2 complex itself, or molecules that interact with the TLR4/MD2 complex such as LPS binding protein (LBP) and membrane bound and soluble CD14, may all be targets of apoCI, which requires further investigation.

The role of CETP in atherosclerosis

The role of CETP in atherosclerosis is still undergoing debate. In this thesis, we show that CETP enhances atherosclerosis in the humanized *APOE*3-Leiden* mouse model.[81] Since the clearance of apoB-containing lipoproteins is attenuated in *APOE*3-Leiden* mice compared to wild-type mice, these data add to the belief that CETP is pro-atherogenic in the context of decreased clearance of apoB-containing lipoproteins. As humans also show a relatively slow clearance of apoB-containing lipoproteins, which is aggravated in atherogenic hyperlipidemia, CETP may thus be expected to be also atherogenic in humans with atherogenic hyperlipidemia. Both increased VLDL cholesterol and decreased HDL cholesterol contributed to increased atherosclerosis in *CETP.APOE*3-Leiden* mice compared to *APOE*3-Leiden* mice. We further investigated the composition and cholesterol efflux inducing properties of HDL from mice of both genotypes. CETP expression in *APOE*3-Leiden* mice led to remodelling of apoE-rich HDL_1 that typically accumulates in these mice. Concomitantly, apoAI and HDL-cholesterol levels decreased by ~25%, and the total cholesterol efflux capacity of the plasma decreased. Whole plasma from *CETP.APOE*3-Leiden* mice was equally effective in inducing ABCA1-mediated cholesterol efflux from macrophages as

compared to plasma from *APOE*3-Leiden* mice. Although the total HDL-cholesterol levels were lower in *CETP.APOE*3-Leiden* mice, the plasma of these mice apparently thus still contains appreciable amounts of small lipid-poor HDL particles, which are the preferred acceptors for ABCA1-mediated efflux. However, plasma from *CETP.APOE*3-Leiden* mice was less effective in inducing SR-BI mediated cholesterol efflux from Fu5AH cells than plasma from *APOE*3-Leiden* mice.[81] This is probably the consequence of the almost complete absence of large apoE-rich HDL_1, which is involved in SR-BI-mediated efflux. ApoE rich HDL_1 particles also accumulate in CETP deficient humans, and induce cholesterol efflux via the ABCG1 pathway.[90] The apoE present on HDL subsequently proved to be a potent LCAT activator in order to rapidly esterify the cholesterol thereby expanding the CE core of HDL, enabling it to take up more cholesterol.[90] It is thus likely that CETP expression in *APOE*3-Leiden* mice also reduces the cholesterol-accepting capacity of plasma by reducing the ABCG1-mediated cholesterol efflux to plasma, and thereby contributed to aggravated atherosclerosis development. In addition, we speculate that CETP expression shifts the pathway of HDL-induced cholesterol efflux from a predominant ABCG1/SR-BI-mediated pathway to an ABCA1-mediated pathway.

The *APOE*3-Leiden* mouse model has already been shown to exert a human-like response to drugs affecting VLDL metabolism, such as statins and fibrates,[91;92] whereas it hardly responds to HDL-increasing therapies. With the introduction of CETP, the *CETP.APOE*3-Leiden* mouse model is now suitable for also testing potential compounds that increase HDL levels, as indicated by pilot experiments with the experimental CETP inhibitor torcetrapib. Corresponding with the effect of torcetrapib treatment in humans,[93] treatment of *CETP.APOE*3-Leiden* mice with torcetrapib increased the level of HDL (W. de Haan *et al*., unpublished observations). At high doses, torcetrapib also led to the formation of large HDL_1 particles in *CETP.APOE*3-Leiden* mice and humans.[93] HDL_1 particles are probably formed upon treatment of humans with CETP inhibitors in general, although this has not yet been reported for for example JTT-705, which is another CETP inhibitor that is currently tested in clinical trials. This CETP inhibitor also induces an increase in HDL-cholesterol, but the size of the HDL was not reported.[94] Cholesterol efflux studies with HDL_1 particles from torcetrapib-treated subjects showed that these particles also exhibited increased ABCG1 mediated cholesterol efflux, yet not

as much as in total CETP deficiency.[95] Nevertheless, recent clinical phase III studies showed more deaths in people suffering from CVD in the group treated with a combination of torcetrapib and atorvastatin than in the group of patients treated with atorvastatin only.[95] It still has to be determined whether this effect is compound-specific, thus related to its effect on increasing the blood pressure, or a consequence of CETP inhibition in general. As it has been reported that CETP inhibition shifts the pathways for cholesterol efflux in the vessel wall from ABCA1 mediated cholesterol efflux to SR-BI/ABCG1 mediated cholesterol efflux, it remains to be addressed to what extent SR-BI/ABCG1 are expressed in the vessel wall relatively to ABCA1, and can really play a role in cholesterol efflux to HDL_1. If ABCA1 is still more important for cholesterol efflux from the vessel wall, HDL1-particles might be less beneficial for the induction of cholesterol efflux from the vessel wall. Future trials will have to show whether other CETP inhibitors, together with classical LDL-lowering drugs such as statins, may constitute a true novel therapy to more effectively treat CVD.

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