Opposing Effects of Apolipoprotein M on Catabolism of Apolipoprotein B–Containing Lipoproteins and Atherosclerosis

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Rationale: Plasma apolipoprotein (apo)M is mainly associated with high-density lipoprotein (HDL). HDL-bound apoM is antiatherogenic in vitro. However, plasma apoM is not associated with coronary heart disease in humans, perhaps because of a positive correlation with plasma low-density lipoprotein (LDL).

Objective: We explored putative links between apoM and very-low-density (VLDL)/LDL metabolism and the antiatherogenic potential of apoM in vivo.

Methods and Results: Plasma apoM was increased ≈2.1 and ≈1.5 fold in mice lacking LDL receptors (Ldlr<−/−) and expressing dysfunctional LDL receptor-related protein 1 (Lrp1<−/−), respectively, but was unaffected in apoE-deficient (ApoE<−/−) mice. Thus, pathways controlling catabolism of VLDL and LDL affect plasma apoM. Overexpression (≈10-fold) of human apoM increased (50% to 70%) and apoM deficiency decreased (≈25%) plasma VLDL/LDL cholesterol in Ldlr<−/− mice, whereas apoM did not affect plasma VLDL/LDL in mice with intact LDL receptors. Moreover, plasma clearance of apoM-enriched VLDL/LDL was slower than that of control VLDL/LDL in mice lacking functional LDL receptors and LRP1, suggesting that apoM impairs the catabolism of VLDL/LDL that occurs independently of the LDL receptor and LRP1. ApoM overexpression decreased atherosclerosis in ApoE<−/− (60%) and cholate/cholesterol-fed wild-type mice (70%). However, in Ldlr<−/− mice the antiatherogenic effect of apoM was attenuated by its VLDL/LDL-raising effect.

Conclusion: The data suggest that defective LDL receptor function leads to increased plasma apoM concentrations, which in turn, impairs the removal of VLDL/LDL from plasma. This mechanism opposes the otherwise antiatherogenic effect of apoM. (Circ Res. 2010;106:1624-1634.)

Key Words: lipid metabolism • atherosclerosis • apolipoprotein M

Plasma lipoproteins play a major role as risk factors for cardiovascular disease. ApoB-containing very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) are proatherogenic whereas the apolipoprotein (apo)A–I–containing high-density lipoprotein (HDL) have antiatherogenic properties.1–3 HDL comprises a heterogeneous class of lipoproteins which in addition to apoA-I contains >40 different proteins one of which is apolipoprotein M (apoM).4 ApoM is produced in liver and kidney.5,6 Liver apoM is secreted to plasma, whereas kidney apoM is secreted into the urine.7 The plasma concentration of apoM is ≈1 μmol/L, ie, comparable to that of apoB (≈2 μmol/L) but lower than that of apoA-I (≈40 μmol/L).8 The major fraction of plasma apoM resides in HDL.9,10 A smaller fraction is present in apoB-containing lipoproteins such as LDL and triglyceride-rich VLDL.9,10 Immuno-depletion studies suggest that ≈5% of plasma HDL and 1 to 2% of plasma LDL particles contain apoM.10 The structure of apoM lacks the amphipathic motifs which normally attach apolipoproteins to the lipid surface of plasma lipoproteins.11 Instead, apoM is bound to plasma lipoproteins via its preserved hydrophobic signal peptide which serves as an anchor for apoM in the lipid moiety of plasma lipoproteins.12,13

Results from 2 independent studies suggest that modest overexpression of human and mouse apoM retards development of atherosclerosis in transgenic mice.14,15 The antiatherogenic effect of apoM overexpression has been ascribed to a stimulatory effect of apoM on preβ-HDL formation.14–16 Preβ-HDL serves as an acceptor of ABCA1-mediated cholesterol efflux from foam cells, and as such promotes reverse cholesterol transport.17 Accordingly, human apoM-containing HDL mediates more cholesterol efflux from macrophage-de-
rived foam cells than apoM-free HDL. Moreover, apoM-containing HDL is a more efficient inhibitor of Cu2+-induced oxidation of LDL than apoM-free HDL.

Even though experimental studies thus imply antiatherogenic potentials of apoM, the plasma apoM concentration did not differ between individuals with cardiovascular disease and control subjects. The reason for this apparent discrepancy is unknown but may relate to a rather strong positive association \((r=0.28\ to 0.42)\) between the plasma apoM and LDL-cholesterol concentrations. Thus, the potential beneficial effect of a high plasma apoM is confounded by the coexistence of elevated LDL which has been consistent in several human studies. The association between plasma LDL cholesterol and apoM could reflect that the plasma apoM concentration is determined by the metabolism of LDL, that apoM affects the plasma LDL concentration, or a combination. It is, however, unknown how the putative link between apoM and apoB metabolism might affect the overall atherogenicity of plasma apoM.

On secretion of apoB48-containing chylomicrons from the intestine or apoB100-containing VLDL from the liver, the triglyceride-rich lipoproteins, which also contain apoE, are subjected to the action of lipoprotein lipase. The apoB48-containing chylomicrons are converted into remnant particles that are rapidly catabolized, primarily in the liver. Liver-derived apoB100-containing VLDL gives rise to LDL and ultimately LDL particles. Normal subjects lose 40 to 50% of their apoB/apoE-containing VLDL/IDL particles from plasma before conversion into LDL. The hepatic clearance of LDL involves binding of apoB100 to the LDL receptor. ApoE also binds to the LDL receptor. Lack of functional LDL receptors causes familial hypercholesterolemia (FH). FH thus results in decreased clearance of plasma LDL, but also increased production rates of apoB-containing lipoproteins. Moreover, FH patients apparently lose few of their apoB/apoE-containing VLDL/IDL particles before conversion into LDL. Despite the decreased fractional catabolic rate of LDL, studies in WHHL-rabbits showed that the LDL-deficient liver still removes large numbers of LDL particles. In the absence of LDL receptors, lipoprotein uptake in the liver appears at least partly dependent on apoE, as judged from studies in mice. Thus, VLDL and LDL can be removed in the liver via apoE-binding to heparan sulfate and LRP1 (LDL receptor–related protein 1). Nevertheless, LRP1-deficient mice as opposed to LDL receptor–deficient mice display no or only minor increases in plasma VLDL/LDL implying that LRP1 is not a major receptor for lipoprotein clearance in the setting of preserved LDL receptor function. Lack of LRP1 in LDL receptor–deficient mice, however, causes some increase in plasma VLDL/LDL suggesting that LRP1 can function as partial backup for the LDL receptor. LRP1 is known about the mechanisms and relative contributions of other non-LDL receptor pathways for lipoprotein uptake in the liver. Studies in genetically modified mice indicate that other receptors such as LRPS and SRB1 as well as proteoglycans may play a role.

In the present study, we have examined links between VLDL/LDL and apoM metabolism by measuring plasma apoM in mice with elevated VLDL/LDL levels attributable to hepatic overproduction (in human apoB transgenic mice \([\text{ApoB-Tg}]\)), decreased LDL receptor–mediated clearance (in LDL receptor–deficient mice \([\text{Ldlr}^−/−]\)), or decreased non-LDL receptor–mediated clearance (in apoE-deficient mice \([\text{ApoE}^−/−]\)) and mice with defect LRP1 \([\text{Lrp1}^n2/n2]\). Moreover, we have assessed the impact of apoM on the metabolism of VLDL/LDL and atherosclerosis using two lines of human apoM transgenic mice (\([\text{ApoM-Tg}]^A\) and \([\text{ApoM-Tg}]^B\)) and apoM deficient mice (\([\text{ApoM}^−/−]\)). The results suggest that defects in the LDL receptor or LRP1 result in increased plasma apoM and that the increased plasma apoM in turn results in delayed clearance of VLDL/LDL particles via non-LDL receptor–mediated pathways. The results also suggest that increased plasma apoM protects against atherosclerosis, but that the beneficial effect of apoM is opposed by the VLDL/LDL-raising effect in the setting of LDL receptor deficiency.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Impact of Perturbed LDL Metabolism on Mouse ApoM**

Mouse apoM was measured with ELISA in wild-type (Wt), \([\text{Ldlr}^−/−]\), \([\text{ApoE}^−/−]\), \([\text{Lrp1}^n2/n2]\), \([\text{Ldlrcyp1}^−/−]\), \([\text{Lrp1}^n2/n2\text{ApoE}^−/−]\), and \([\text{ApoB-Tg}]^B\) mice. ApoM expression in human apoB transgenic mice was inhibited in vivo with an apoB antisense oligonucleotide. The \(d<1.063\) and \(d>1.063\) g/mL plasma lipoproteins were separated with fixed density ultracentrifugation. Plasma lipids were determined with enzymatic assays.

**In Vivo Metabolism of ApoM and ApoM-Containing VLDL/LDL**

The exchange of apoM between lipoproteins was examined in vivo by injecting human apoM-containing VLDL/LDL or HDL into \([\text{Ldlr}^−/−]\) mice and \([\text{Wt}]\) mice. Human apoM in individual lipoprotein fractions was determined by gel filtration of recipient mouse plasma and human apoM ELISA and Western blotting of isolated fractions. The turnover of apoM-enriched VLDL/LDL versus apoM-poor VLDL/LDL was determined by iodination of isolated VLDL/LDL from \([\text{ApoM-Tg}]^\text{A}\) \([\text{Ldlr}^−/−]\) and \([\text{Ldlr}^−/−]\) mice with \(^{125}\text{I}\) and \(^{131}\text{I}\). The labeled lipoproteins were co-injected into \([\text{Ldlr}^−/−]\) and \([\text{Lrp1}^n2/n2]\) \([\text{Ldlr}^−/−]\) mice and the plasma decay followed by \(\gamma\)-counting of plasma (10 \(\mu\text{L}\)) from the recipient mice.

**Impact of ApoM on Lipoproteins and Atherosclerosis**

\([\text{ApoM}^−/−]\), \([\text{ApoM-Tg}]^B\), and \([\text{ApoM-Tg}]^A\) mice were crossed with \([\text{Ldlr}^−/−]\) mice, \([\text{ApoM-Tg}]^B\) mice were crossed with \([\text{ApoE}^−/−]\) mice and

ApoM-Tg mice were fed a cholic-acid/cholesterol-enriched diet to induce hyperlipidemia. Plasma lipoproteins were separated with gel filtration chromatography; pooled fractions were used for Western blotting with antibodies against mouse apolipoproteins. Aortic atherosclerosis was quantified as en face lesion areas and/or oil-red O staining lesions in histological cross sections of the aortic root.

Results

Deficiency of the LDL Receptor or LRP1 Increases Plasma ApoM

To assess the impact of perturbed VLDL/LDL metabolism on plasma apoM concentrations, we quantified apoM with ELISA (Online Figure I) in Wt mice and in mice with impaired clearance of apoB- and apoE-containing lipoproteins.

Compared with Wt mice, plasma apoM was increased \( \approx 110\% \) (\( P<0.0005 \)) in \( Ldlr^{−/−} \) mice with elevated VLDL/LDL (Figure 1A and 1B). Mice with a knock-in mutation in the NPxYxxL motif of LRP1 designed to attenuate LRP1-mediated lipoprotein internalization (\( Lrp1^{n2/n2} \)) also had increased plasma apoM \( \approx 50\% \) (\( P<0.005 \)) despite less pronounced elevation of the plasma cholesterol concentration (Figure 1A and 1B). Combined deficiency of the LDL receptor and LRP1 caused a further increase of plasma apoM (Figure 1B). In contrast, total plasma apoM was not significantly increased in \( ApoE^{−/−} \) mice irrespective of the pronounced elevation of VLDL/LDL cholesterol (Figure 1A and 1B). Moreover, apoE-deficiency completely abolished the increase of plasma apoM in \( Lrp1^{n2/n2} \) mice (Figure 1B).

In a subset of the mice, we used ultracentrifugation of plasma to assess to what extent the increase in total plasma apoM in \( Ldlr^{−/−} \) and \( Lrp1^{n2/n2} \) mice reflected increased apoM in the VLDL/LDL (\( d<1.063 \text{ g/mL} \)) or in the HDL (\( d>1.063 \text{ g/mL} \)) fraction. Compared to Wt mice, apoM in the VLDL/LDL fraction was significantly increased in \( Ldlr^{−/−} \) and \( Lrp1^{n2/n2} \) mice (Online Figure II, A). Nevertheless, the major portion of plasma apoM was recovered in HDL (Online Figure II, A) and the increase of total plasma apoM in \( Ldlr^{−/−} \) and \( Lrp1^{n2/n2} \) mice therefore mainly reflected increases of HDL-associated apoM (Online Figure II, A). Nevertheless, the results, which were reiterated by Western blotting against apoM (data not shown), suggest that deficiency of LDL receptors or impairment of LRP1 increases HDL-associated apoM in an apoE-dependent manner. Additional Western blotting examinations suggested that HDL-associated apoE like HDL-associated apoM is increased in \( Ldlr^{−/−} \) mice (Online Figure II, B). This result further supports the notion that LDL receptor deficiency affects the

Figure 1. Deficiency of LDL receptors or LRP1 increases plasma apoM. Plasma cholesterol (A) and apoM (B) in Wt, \( Ldlr^{−/−} \), ApoE \( ^{−/−} \), \( Lrp1^{n2/n2} \), \( Lrp1^{n2/n2} Ldlr^{−/−} \), and \( Lrp1^{n2/n2} ApoE^{−/−} \) female mice; \( Lrp1^{n2/n2} \) mice express a dysfunctional LRP1. \( *P<0.005 \) or \( **P<0.0005 \) compared with Wt mice. The concentration of apoM is expressed as percentage of the level in a plasma pool from C57B6/J mice.

Figure 1 (Continued). \( Lrp1^{n2/n2} \) ApoE \( ^{−/−} \) female mice; \( Lrp1^{n2/n2} \) mice express a dysfunctional LRP1. \( P<0.005 \) or \( *P<0.0005 \) compared with Wt mice. The concentration of apoM is expressed as percentage of the level in a plasma pool from C57B6/J mice. C, Plasma apoM in ApoB-Tg (left filled bar) and Wt littermate control male mice (left open bar). In a separate experiment, ApoB-Tg mice received an intraperitoneal injection of an apoB-antisense oligonucleotide which lowered plasma LDL-cholesterol (Online Figure III). Plasma apoM was determined before (right open bar) and 4 days after the injection (right filled bar). Values are means±SEM. The number of mice in each group is indicated within the bars.
Overexpression of ApoB Decreases Plasma ApoM

LDL receptor deficiency results in impaired clearance of apoB-containing lipoproteins but also in increased production rates.\textsuperscript{23,24} We assessed the impact of elevated plasma LDL-cholesterol exclusively caused by increased hepatic production on the concentration of plasma apoM. Heterozygous human ApoB-Tg mice with increased hepatic apoB-secretion displayed increased LDL-cholesterol (Online Figure III, A and B) but decreased plasma apoM concentrations that were \(\approx 50\%\) of the level in littermate Wt mice (Figure 1C). We suspect that the reduction of plasma apoM by apoB overexpression may reflect (1) that apoM bound to LDL has a shorter half-life than apoM bound to plasma HDL because of the effective LDL receptor–mediated clearance of plasma LDL, and (2) that a larger fraction of plasma apoM will attach to LDL in ApoB-Tg mice with markedly elevated plasma LDL than in Wt mice with almost absent plasma LDL. Accordingly, lowering of the plasma LDL-cholesterol with an apoB-antisense oligonucleotide that targets both mouse and human apoB mRNA (Online Figure III, A and B) reversed plasma apoM concentrations to normal in ApoB-Tg mice (Figure 1C).

Exchange of ApoM Between Plasma Lipoproteins

In Vivo

The predominant increase of HDL-associated apoM in mice with defect LDL receptor and/or LRP1 function as well as the lowering of apoM in ApoB-Tg mice could reflect either direct effects of a perturbed metabolism of apoB-containing lipoproteins on apoM-containing HDL particles or exchange of apoM between HDL and VLDL/LDL particles (or a combination). We examined whether apoM can exchange from HDL to VLDL/LDL particles in mice by injecting human apoM-enriched HDL (from ApoM-Tg\textsuperscript{15} mice) into Ldlr\textsuperscript{-/-} mice with high VLDL/LDL cholesterol or Wt mice. Already 5 minutes after the injection a significant fraction of the initially HDL-associated apoM was recovered in the VLDL/LDL fraction of recipient Ldlr\textsuperscript{-/-} mice (Figure 2A and 2B) but remained in the HDL fraction of recipient Wt mice (Online Figure IV, A). We also examined the distribution of VLDL/LDL-associated apoM to HDL particles by injecting human apoM-enriched VLDL/LDL into recipient Wt or Ldlr\textsuperscript{-/-} mice. In Wt recipient mice, the vast portion of the VLDL/LDL-derived human apoM was recovered in HDL after 5 minutes (Figure 2A and 2C). A less pronounced exchange of human apoM to HDL was seen in Ldlr\textsuperscript{-/-} recipient mice (Online Figure IV, B). The redistribution of apoM among the lipoproteins of the recipient mice was consistent also when analyzing plasma from the recipient mice at 180 minutes after the injections (data not shown). These results show that apoM can rapidly exchange between plasma lipoproteins.

Figure 2. ApoM exchange rapidly between lipoprotein particles in vivo. Pooled plasma with human apoM-enriched HDL from ApoM-Tg\textsuperscript{15} and purified VLDL/LDL from ApoM-Tg\textsuperscript{15}Ldlr\textsuperscript{-/-} were injected intravenously into 3 recipient Ldlr\textsuperscript{-/-} mice and 3 recipient Wt mice, respectively. A, The lipoprotein distribution of human apoM in injected material and plasma of recipient mice was assessed with Western blotting; VLDL, LDL1, LDL2, HDL, and protein fractions were pooled gel filtration fractions (shown in Figure IV, A). We also examined the distribution of VLDL/LDL-associated apoM to HDL particles by injecting human apoM-enriched VLDL/LDL into recipient Wt or Ldlr\textsuperscript{-/-} mice. In Wt recipient mice, the vast portion of the VLDL/LDL-derived human apoM was recovered in HDL after 5 minutes (Figure 2A and 2C). A less pronounced exchange of human apoM to HDL was seen in Ldlr\textsuperscript{-/-} recipient mice (Online Figure IV, B). The redistribution of apoM among the lipoproteins of the recipient mice was consistent also when analyzing plasma from the recipient mice at 180 minutes after the injections (data not shown). These results show that apoM can rapidly exchange between plasma lipoproteins.

ApoM Increases Plasma VLDL- and LDL-Cholesterol in Ldlr\textsuperscript{-/-} Mice

In Wt mice, overexpression of apoM doubles the plasma concentration of apoE in HDL and increases HDL cholesterol by \(\approx 20\%\).\textsuperscript{14} We have, however, not seen any in- or decrease of plasma VLDL/LDL-cholesterol in Wt mice that overexpress human apoM or lack endogenous apoM.\textsuperscript{14} Nevertheless, it became apparent that apoM affects the metabolism of apoB- and apoE-containing lipoproteins when we crossed Ldlr\textsuperscript{-/-} and ApoE\textsuperscript{-/-} mice with a line of human apoM transgenic mice that overexpress apoM \(\sim 10\)-fold (ApoM-Tg\textsuperscript{14}).

The plasma cholesterol and triglyceride concentrations were increased in ApoM-Tg\textsuperscript{14}Ldlr\textsuperscript{-/-} mice compared to
littermate Ldlr−/− control mice and the difference in plasma cholesterol was further accentuated to ≈70% (37.4 ± 4.4 versus 21.9 ± 2.3 mmol/L, *P < 0.01) when the mice were fed a Western-type diet. Gel filtration chromatography showed that the increase in plasma cholesterol reflected increases of both VLDL- and LDL-cholesterol whereas HDL-cholesterol appeared unaffected (Figure 3A). Western blot analysis of lipoprotein fractions from the gel filtration column showed that apoM overexpression increased the content of apoE in HDL (Figure 3B). Notably, the effect of apoM overexpression on apoB48 in VLDL and LDL (Figure 3F). ApoM deficiency also decreased apoE, both in LDL and HDL (Figure 3F). ApoM−/−Ldlr−/− mice displayed no significant change in the total plasma cholesterol concentration or plasma gel filtration profile as compared with Ldlr−/− mice (data not shown).

### Impaired Clearance of ApoM-Enriched VLDL/LDL in Ldlr−/− Mice

The effects of apoM overexpression and apoM deficiency on VLDL/LDL in Ldlr−/− mice could reflect that the presence of apoM in VLDL/LDL impairs plasma clearance of VLDL/LDL via non-LDL receptor–mediated pathways. To examine this possibility, VLDL/LDL particles from ApoM-Tg4Ldlr−/− mice (which are enriched in human apoM, Figures 2A and 3B) and VLDL/LDL particles from control Ldlr−/− mice (with less apoM, Figure 3B) were labeled with, respectively, 125I and 131I (or vice versa) and coinjected into recipient Western-type diet fed Ldlr−/− mice. The plasma clearance of *I-VLDL/LDL from ApoM-Tg4Ldlr−/− mice was slower than that of *I-VLDL/LDL from Ldlr−/− control mice (data not shown). To further test whether delayed clearance might be dependent on LRP1, we repeated the experiment (Figure 4A) and in addition compared the clearance of human apoM-enriched VLDL/LDL with that of apoM-poor VLDL/LDL in mice lacking both the LDL receptor and functional LRP1. The clearance of the apoM-enriched VLDL/LDL was lower than that of apoM poor VLDL/LDL both in Ldlr−/− and in Lrp1−/− mice (Figure 4B).

ApoM readily exchanges between plasma lipoprotein (vide supra). Hence, a larger fraction of the radioactivity from apoM-enriched VLDL/LDL than from apoM-poor VLDL/L

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### Table. Effect of ApoM Overexpression or Deficiency on Plasma Cholesterol and Triglycerides

<table>
<thead>
<tr>
<th>Sex</th>
<th>Cholesterol (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
<th>Western-Type Diet</th>
<th>Cholesterol (mmol/L)</th>
<th>Triglycerides (mmol/L)</th>
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<tr>
<td>Ldlr−/−</td>
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<td>24.6 ± 1.3*</td>
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<tr>
<td>ApoM−/−ApoE−/−</td>
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<td>13.6 ± 0.6§</td>
<td>1.5 ± 0.2§</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

**Nonfasting plasma lipids (means ± SEM) were measured at 4–5 weeks of age when all mice were on a chow diet and at 20 weeks of age after feeding a Western-type diet for 16 weeks unless otherwise indicated. *P < 0.05 and †P < 0.08 compared to the appropriate littermate control mice. ‡Mice with a C57B6/J background were fed a cholate/cholesterol-containing diet for 16 weeks. §ApoE−/− mice were fed a chow diet for 16 weeks (similar results were obtained in 5-week-old mice). ND indicates not determined.**
LDL was recovered in HDL-sized lipoproteins of the recipient mice (Online Figure V, A). Importantly, however, like total plasma radioactivity (Figure 4), the VLDL/LDL-associated radioactivity from apoM-poor VLDL/LDL was removed faster than that from apoM-enriched VLDL/LDL. Thus, after 6 hours 37% and 34% of the initial VLDL/LDL-associated radioactivity from human apoM-poor VLDL/LDL and 25% and 26% of the initial VLDL/LDL-associated radioactivity from apoM-poor VLDL/LDL particles remained in the plasma VLDL/LDL fraction of the Ldlr−/− and Lrp1ap2/n2 Ldlr−/− recipient mice, respectively (Online Figure V, B). Thus, the results are compatible with the notion that delayed clearance of VLDL/LDL by an LDL receptor- and LRPI-dependent pathway contributes to the increased plasma VLDL/LDL cholesterol in ApoM-Tg4 Ldlr−/− mice.

**Effect of ApoM on Atherosclerosis in Ldlr−/− Mice**

We previously observed that a mild (≈2-fold) overexpression of human apoM (ie, in ApoM-Tg4 mice) results in ≈39% smaller atherosclerotic lesion areas in Ldlr−/− female mice. In that study, the female ApoM-Tg4 Ldlr−/− and Ldlr−/− controls had essentially identical plasma lipoprotein profiles, suggesting that ≈2-fold overexpression of apoM has minor effects on VLDL/LDL catabolism in Ldlr−/− female mice. We have subsequently examined plasma lipoproteins and atherosclerosis in male ApoM-Tg4 Ldlr−/− and Ldlr−/− controls. In accordance with the findings in ApoM-Tg4 Ldlr−/− mice, male ApoM-Tg4 Ldlr−/− mice had ≈28% increased total plasma cholesterol levels compared with Ldlr−/− littermate controls on a Western-type diet (Table). Despite the higher plasma cholesterol concentration in ApoM-Tg4 Ldlr−/− mice, there was no difference in atherosclerotic plaque areas between male ApoM-Tg4 Ldlr−/− and Ldlr−/− littermate controls (Figure 5A).

We next examined the impact of extensive (≈10-fold) apoM overexpression on atherosclerosis in ApoM-Tg4 Ldlr−/− mice. The atherosclerotic plaque areas did not differ between male ApoM-Tg4 Ldlr−/− and Ldlr−/− controls (Figure 5B) despite the ≈70% higher plasma cholesterol concentration in the ApoM-Tg4 Ldlr−/− (Table). These data are compatible with the results in ApoM-Tg4 Ldlr−/− mice suggesting that overexpression of human apoM opposes the
proatherogenic effect of an elevated VLDL/LDL-cholesterol concentration in male Ldlr<sup>−/−</sup> mice. In female mice, however, both the plasma cholesterol concentration and atherosclerotic plaque areas were ≈50% higher in ApoM-Tg<sup>apoM<sup>+/−</sup>Ldlr<sup>−/−</sup></sup> mice compared with Ldlr<sup>−/−</sup> littermate controls (P<0.005). Thus, ≈10-fold overexpression of human apoM overexpression could not abolish the marked increase in plasma VLDL/LDL in female Ldlr<sup>−/−</sup> mice.

To examine the impact of endogenous apoM on atherosclerosis in Ldlr<sup>−/−</sup> mice, we compared ApoM<sup>−/−</sup>Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup> mice. Deficiency of apoM, which decreased plasma VLDL- and LDL-cholesterol (Table), reduced atherosclerotic plaque formation in female Ldlr<sup>−/−</sup> mice (Figure 5D). The mean atherosclerotic plaque area of mice that were heterozygous for the apoM targeted gene locus (ApoM+/−Ldlr<sup>−/−</sup>) were larger than that in ApoM<sup>−/−</sup>Ldlr<sup>−/−</sup> mice but did not differ significantly from that in the Ldlr<sup>−/−</sup> control mice (Figure 5D).

**Effect of ApoM Overexpression on Atherosclerosis in Mouse Models With Intact LDL Receptors**

The results obtained in Ldlr<sup>−/−</sup> mice with altered apoM expression suggested that the antiatherogenic effect of raising apoM was opposed by apoM increasing plasma VLDL/LDL-cholesterol in the setting of LDL receptor deficiency. We therefore predicted that overexpression of apoM would substantially decrease atherosclerosis in hypercholesterolemic mice with intact LDL receptors. Support of this idea was obtained from two additional studies. First, we fed a proatherogenic cholate/cholesterol-enriched diet to ApoM-Tg<sup>N</sup> and Wt littermate female mice. ApoM overexpression did not significantly affect the extent of hypercholesterolemia in the cholate/cholesterol-fed mice. The plasma cholesterol and triglyceride concentrations were 7.0 ± 0.3 and 0.1 ± 0.0 mmol/L, respectively in ApoM-Tg<sup>N</sup> (n=14) mice and 6.1 ± 0.4 and 0.1 ± 0.0 mmol/L, respectively, in Wt mice (n=9) (Table) and the gel filtration profiles of plasma lipoproteins were similar in the two groups (Online Figure VI). Because these mice have intact apoE expression, the latter finding argues against the possibility that the effect of apoM on plasma VLDL/LDL in Ldlr<sup>−/−</sup> mice depends on apoE. Remarkably, there was ≈70% less atherosclerosis in ApoM-Tg<sup>N</sup> compared with Wt control mice (Figure 6, left). Second, we examined atherosclerotic lesions in 16-weeks-old ApoM-Tg<sup>H</sup>ApoE<sup>+/−</sup> and ApoE<sup>+/−</sup> littermate control mice with similar plasma lipoprotein levels (Figure 3C). The ApoM-Tg<sup>H</sup>ApoE<sup>+/−</sup> mice developed ≈60% less atherosclerosis than the ApoE<sup>+/−</sup> controls (Figure 6, right).

**Discussion**

Previous studies have highlighted putative roles of apoM in HDL metabolism. ApoM has potential antiatherogenic functions, ie, stimulation of preβ-HDL formation and potential contribution to the antioxidative effect of HDL. In the present study, we used genetically modified mice to demonstrate hitherto unknown links between the metabolism of apoM and VLDL/LDL which affect the antiatherogenic potential of plasma apoM. The major findings were that (1) plasma apoM concentrations are increased when VLDL/LDL catabolism is decreased because of the absence of LDL receptors or fully functional LRP1; (2) the increase of plasma apoM is dependent on apoE and mainly occurs in HDL; (3) apoM readily exchanges between plasma lipoproteins in vivo;
(4) overexpression or deficiency of apoM increases or decreases, respectively, the plasma levels of apoE and apoB48-containing VLDL/LDL in Ldlr−/− mice even though the effect of modest apoM overexpression may be more pronounced in female than in male mice, whereas apoM overexpression has no effect on VLDL/LDL levels in mice with hypercholesterolemia attributable to absence of apoE or attributable to feeding a cholate/cholesterol-enriched diet; (5) the clearance of apoM-enriched VLDL/LDL likely is decreased in mice lacking both LDL receptors and functional LRP1; (6) apoM retards atherogenesis in ApoE−/− and cholate/cholesterol-fed mice, whereas the antiatherogenic effect is attenuated in Ldlr−/− mice; and (7) the antiatherogenic effect of apoM was more pronounced in male than in female mice in the setting of LDL receptor deficiency.

The crossroads between the metabolic pathways of plasma HDL and VLDL/LDL are incompletely understood. HDL-associated apoM was increased in Ldlr−/− and Lrp1n2n2 mice indicating that the activity of the receptors, known to control the catabolism of VLDL and LDL, also affects the catabolism of apoM-containing HDL particles. The impact of the LDL receptor and LRP1 on plasma apoM was a surprise because apoA-I and HDL-cholesterol are largely unaffected by LDL receptor deficiency. In fact, HDL-cholesterol and apoA-I are often slightly decreased in patients with homozygous FH.37 Because the effect of defect LRP1 on plasma apoM was
ApoE was clearly increased in LDL receptor–deficient mice. Overexpression of apoM in −/− mice abolishes the effect of apoE on plasma VLDL/LDL levels in mice with intact LDL receptors. Oil red O staining lesion areas were determined in 6 to 8 histological cross-sections of the aortic root in 20-week-old ApoM-Tg" (filled bars, left) and WT (open bars, left) female mice that had been fed a cholate-enriched (0.5%) and cholesterol-enriched (1.25%) diet from 4 weeks of age and in 16-week-old ApoM-Tg"ApoE−/− (filled bars, right) and ApoE−/− (open bars, right) chow-fed mice. Values are means±SEM. The number of mice in each group is indicated within the bars.

Figure 6. ApoM reduces atherosclerosis in mice with intact LDL receptors. Oil red O staining lesion areas were determined in 6 to 8 histological cross-sections of the aortic root in 16-week-old ApoM-Tg" (filled bars, left) and WT (open bars, left) female mice that had been fed a cholate-enriched (0.5%) and cholesterol-enriched (1.25%) diet from 4 weeks of age and in 16-week-old ApoM-Tg"ApoE−/− (filled bars, right) and ApoE−/− (open bars, right) chow-fed mice. Values are means±SEM. The number of mice in each group is indicated within the bars.

The results thus provide new insight into human physiology by offering an explanation for the strong positive association between plasma LDL-cholesterol and apoM concentrations in humans. In humans, the fractional catabolic rate of LDL is an important determinant of the normal interindividual variation in plasma LDL-cholesterol concentrations. The present investigations in mice demonstrate that the plasma apoM concentration also is markedly affected by the LDL receptor pathway. Thus, it is likely that a decreased plasma concentration of VLDL/LDL will increase both plasma apoM and LDL-cholesterol and vice versa in humans.

We discovered a marked effect of apoM on VLDL/LDL metabolism when ApoM-Tg" and ApoM−/− mice were crossed with Ldlr−/− mice. Overexpression of apoM increased and deficiency of apoM decreased the plasma VLDL/LDL-cholesterol concentration in Ldlr−/− mice. ApoM overexpression did not significantly affect VLDL/LDL-cholesterol in ApoE−/− mice even though their plasma VLDL/LDL-cholesterol concentrations were increased to the same extent as in Ldlr−/− mice. As such, the results could be interpreted as if the effect of apoM on plasma VLDL/LDL levels is dependent on apoE. However, there was no effect of apoM overexpression on plasma VLDL/LDL cholesterol in cholate/cholesterol-fed or chow-fed Wt mice with intact apoE implying that the effect of apoM on plasma VLDL/LDL only is significant in the absence of intact LDL receptors. If these data can be extrapolated to humans, they imply that the association between plasma apoM and LDL cholesterol levels in normal humans with intact LDL receptors for the largest part reflects an effect of plasma LDL clearance on apoM (as discussed above) rather than effects of apoM on plasma LDL metabolism.

Because apoM did not affect plasma VLDL/LDL in ApoE−/− or Wt mice, we suspect that apoM does not affect hepatic lipoprotein secretion. We therefore performed turnover studies with radio-iodinated VLDL/LDL to examine whether apoM might affect VLDL/LDL catabolism. The removal from plasma was slower for labeled apoM-enriched VLDL/LDL (isolated from ApoM-Tg"Ldlr−/− mice) than for labeled VLDL/LDL from Ldlr−/− mice both in Ldlr−/− and Lrp12/2/2Ldlr−/− mice. Even though the results thus offer support to the idea that apoM impairs the clearance of VLDL/LDL via non-LDL receptor and LRP1 mediated pathways, the data should be interpreted with caution. Hence, in agreement with the observation that apoM rapidly exchanges between lipoprotein fractions, a significant portion of the radioactivity from labeled apoM-enriched VLDL/LDL appeared in the HDL fraction. Nevertheless, the VLDL/LDL associated radioactivity was more slowly removed from plasma when comparing labeled apoM-enriched VLDL/LDL with labeled apoM-poor VLDL/LDL. The LDL receptor and LRP1-independent pathway(s) for VLDL/LDL clearance that may be hindered by apoM is unknown. It could involve scavenger receptors event though we have not seen any effects of murine SRB1 deficiency on plasma apoM levels (C Christoffersen, LB Nielsen, unpublished data, 2010). What might be the physiological meaning of apoM decreasing VLDL/LDL (and possibly apoE-containing HDL) clearance via non-LDL receptor-mediated pathways? ApoM is a lipocalin with ability to bind a small lipophilic ligand(s). Recombinant apoM binds retinol and retinoic acid, but the physiological ligand of apoM is unknown. Normally, the plasma half-life of VLDL/LDL is very rapid compared with LDL and HDL. Thus, we speculate that the inhibitory effect of apoM on lipoprotein clearance may have evolved to delay removal of the lipoproteins that carry apoM, thus serving to maintain apoM (and its ligand) in the circulation.

In contrast to the situation in normal humans with intact LDL receptors and irrespective of the physiological function, the present findings imply that apoM may be a determinant of plasma VLDL and LDL levels in patients with FH. Thus, based on the findings in mice it is conceivable that FH patients have increased plasma apoM concentrations that in turn increases plasma VLDL/LDL. Further elucidation of this hypothetical scenario obviously requires studies that link apoM and VLDL/LDL cholesterol levels in FH individuals. At this stage, there are no known means to manipulate plasma apoM in humans. Recent studies have, however, identified...
common polymorphisms in the apoM promoter region that may affect apoM plasma levels. It would be interesting to determine whether genetically induced changes in apoM expression might modulate the hypercholesterolemic response to LDL receptor or apoB3500 mutations in humans.

The present studies of hypercholesterolemic mice with altered apoM expression further establish that apoM is capable of retarding the development of atherosclerosis, at least in mice. Remarkably, however, in the setting of LDL receptor deficiency the antiatherogenic effect of apoM was blunted by the proatherogenic effect of raising VLDL/LDL, especially in female mice. This may reflect that the antiatherogenic effect of apoM is more pronounced in male than in females. Notably, however, apoM overexpression did reduce atherosclerosis in female ApoE−/− and cholate/cholesterol-fed female mice. Thus, further studies are needed to resolve the putative gender specific effects of apoM in atherogenesis. The antiatherogenic effect of raising apoM in ApoE−/− and cholate/cholesterol-fed mice agrees with previous in vitro findings showing that apoM increases the antiatherogenic properties of HDL by promoting preβ-HDL formation, increasing the ability of HDL to mobilize cholesterol from foam cells and inhibit oxidation of LDL. It is unknown which of these mechanisms are most important in vivo.

A recent study showed that apoM plasma levels indeed are positively related to preβ-HDL formation in humans. Nevertheless, the mean plasma apoM concentration was almost identical in patients with cardiovascular disease and controls. It is possible that this lack of association between plasma apoM and cardiovascular risk in humans reflects that a high plasma apoM concentration is linked with high plasma LDL residence times and concentrations. As such, the lack of predictive power of plasma apoM in relationship to human cardiovascular disease does not rule out that increasing plasma apoM might have beneficial effects in human atherogenesis, at least in non-FH patients.

Acknowledgments
We thank Karen Rasmussen, Charlotte Wandel, Maria Kristensen, and Tina Axen for excellent technical assistance and Dr Ellen Marie Straaup (Sanitatis Pharma, Denmark) for the apoB-antisense oligonucleotide.

Sources of Funding
The study was supported by grants from the Danish Research Council (to L.B.N.); the Danish Heart Foundation (to C.C.); the Novo Nordisk Foundation (to L.B.N.); Rigshospitalet, University of Copenhagen (to L.B.N.); the Swedish Research Council (no. 07143) (to B.D.), the Swedish Heart-Lung Foundation (to B.D.), the Wallenberg Foundation (to B.D.), and Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen) (to P.G.).

Disclosures
None.

References
21. Goldstein JL, Brown MS, Anderson RG, Russell DW, Schneider WJ, Ridker P, Oram EC, Boston RC, Tada N, Mochizuki S, Defesche JC, Wilson JM, Rader DJ. Complete deficiency of the low-density lipoprotein receptor is associ-
A novel receptor that mediates a distinct pathway for ligand catabolism.


What New Information Does This Article Contribute?

There is a tight link between the metabolism pathways of VLDL/LDL and of HDL-associated apoM.

The effect of apoM on plasma VLDL/LDL opposes the anti-atherogenic potential of apoM in the setting of LDL receptor deficiency.

Plasma apoM is mainly associated with HDL. Nevertheless, studies in humans consistently have shown a positive correlation between the plasma apoM and LDL concentrations. We used a panel of genetically modified mice to investigate possible links between apoM and VLDL/LDL metabolism and how they may affect the anti-atherogenic potential of apoM. HDL-associated apoM was markedly increased when plasma VLDL/LDL was increased because of lack of LDL or LRP1 receptors; this effect was dependent on apoE. Furthermore, apoM delayed the clearance of VLDL/LDL in mice with LDL receptor deficiency but not in mice with intact LDL receptors. A series of 5 different atherosclerosis studies in different mouse models showed a marked anti-atherogenic effect of apoM in models with intact LDL receptors. In contrast, the beneficial effect of apoM was opposed by the VLDL/LDL-raising effect in LDL receptor-deficient mice. This study is the first to show significant links between the catabolism of VLDL/LDL and a predominantly HDL-associated apolipoprotein.
Opposing Effects of Apolipoprotein M on Catabolism of Apolipoprotein B–Containing Lipoproteins and Atherosclerosis
Christina Christoffersen, Tanja Xenia Pedersen, Philip L.S.M. Gordts, Anton J.M. Roebroek, Björn Dahlbäck and Lars Bo Nielsen

_Circ Res._ 2010;106:1624-1634; originally published online April 1, 2010;
doi: 10.1161/CIRCRESAHA.109.211086

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENTAL MATERIAL

Detailed methods

Animals. Mice were housed at the Panum Institute (University of Copenhagen, Copenhagen, Denmark) in a temperature-controlled facility with a 12-hour dark/light cycle and fed standard chow (Altromin no.1314, Brogaarden, Gentofte, Denmark) until 4-5 weeks of age. ApoM-Tg\(^{1}\), ApoM-Tg\(^{2}\), or ApoM\(^{-}\) male mice (backcrossed more than 7 times with C57B6/J mice\(^{1}\)) were crossed with Ldlr\(^{-}\) or ApoE\(^{-}\) female mice on a C57B6/J background (from Taconic Europe or The Jackson Laboratory) to obtain ApoM-Tg\(^{1}\)Ldlr\(^{-}\), ApoM-Tg\(^{2}\)Ldlr\(^{-}\), ApoM\(^{-}\)Ldlr\(^{-}\), ApoM\(^{-}\)Ldlr\(^{-}\), ApoM-Tg\(^{1}\)ApoE\(^{-}\), and litter-mate control Ldlr\(^{-}\) and ApoE\(^{-}\) mice. Female Lpr\(^{I262}\) mice were produced and housed in Leuven, Belgium.\(^{2}\) Female Wt, Ldlr\(^{-}\), ApoE\(^{-}\) and the double mutant mice used for plasma apoM measurements were housed in the same facility. ApoB-Tg mice\(^{3}\) were purchased from Taconic Europe. Genotyping of ApoM-Tg, ApoM\(^{-}\), and Ldlr\(^{-}\) mice was done as previously described.\(^{1}\) ApoB-Tg mice were identified with a human apoB-specific plasma ELISA. ApoE\(^{-}\) mice were genotyped according to protocols from the Jacksons Laboratory. Blood samples were taken from the orbital venous plexus. Plasma was isolated by centrifugation at 3000 rpm for 10 min at 4 °C and stored at –80 °C. All procedures were approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

To study the effect of apoM on atherosclerosis, Ldlr\(^{-}\) mice where fed a cholate–free Western type diet with 0.3 % cholesterol and 4.25 % fat (D01061402, Brogaarden). Hypercholesterolemia and atherosclerosis in Wt and ApoM-Tg\(^{1}\) mice were induced with a cholate (0.5 %)/cholesterol (1.25 %)-enriched diet (C13002, Diet Research, New Brunswick, USA). The diets were started at 4-5 weeks of age and the mice were sacrificed 16 weeks later. ApoE\(^{+}\) and ApoM-Tg\(^{2}\)ApoE\(^{+}\) mice were maintained on standard chow until sacrifice at 16 weeks of age.

To reduce apoB expression in vivo, we used a 14-mer single-stranded antisense oligonucleotide made from locked nucleic acid (LNA) and generously provided by Santaris Pharma A/S, Denmark.\(^{4}\) The antisense oligonucleotide which targets both mouse and human apoB was injected (5 mg/kg) intraperitoneally into ApoB-Tg mice 4 days prior to collecting blood for plasma analyses.

Mouse apoM ELISA. Costar 96-well plates (Biotech Line A/S, Slangerup, Denmark) were coated with 16 µg/well of HiTrap-Protein-A-HP column (GE Healthcare Life Sciences, Broendby, Denmark)-purified polyclonal anti-mouse apoM IgG.\(^{5}\) The plates were quenched with 1 % BSA and washed with PBS added 0.1 % Triton-X-100 (Sigma Aldrich Danmark A/S, Broendby, Denmark). Mouse plasma were diluted 1:750 with PBS containing 1 % BSA and 0.1 % triton-X100 was mixed with 33.6 ng recombinant mouse apoM\(^{6}\) that had been biotinylated using reagents from Pierce (VWR-Bie & Berntsen, Herlev, Denmark). The mixture was added to the plate and left for ~16 hours at 20-24 °C. Bound biotinylated recombinant mouse apoM was detected with streptavidin-avidin-horseradish peroxidase (DAKO A/S, Glostrup, Denmark) and o-phenylenediamine (OPD) (DAKO A/S) according to the manufactures instructions. A dilution series made from a plasma pool from C57B6/J mice was included on each plate. Results are expressed as percent of the concentration of apoM in the plasma pool. The variation coefficient of the assay was ~16 %.

Plasma lipoproteins. Plasma lipid determinations, gel filtration, and ultracentrifugations were performed as previously described.\(^{1}\) All plasma lipid measurements were done in duplicate.

Western blotting. Aliquots of pooled fractions gel filtration fractions were separated on 12 % SDS-PAGE gels and western blotting visualizing mouse apoM, apoA-I, and apoE and human apoM was done as described.\(^{1}\) Mouse apoB was detected with a rabbit anti-mouse apoB48/100 antibody (1:1000) (Nordic Biosite Aps, Copenhagen, Denmark).

Plasma metabolism of VLDL/LDL. To compare the plasma removal of VLDL/LDL from ApoM-Tg\(^{2}\)Ldlr\(^{-}\) and Ldlr\(^{-}\) mice, the d < 1.063 g/mL lipoprotein fraction (i.e. VLDL+LDL), was isolated by ultracentrifugation from 300 µl plasma pooled from cholesterol-fed ApoM-Tg\(^{2}\)Ldlr\(^{-}\) or Ldlr\(^{-}\) female mice.\(^{1}\) The purified VLDL/LDL fractions were dialyzed against 10 mM PBS with 0.1 g/l Na\(_{2}\)EDTA at 4 °C and

1
stored at –80 °C. Protein concentrations were measured with the Pierce BCA protein assay kit (VWR-Bie & Berntsen A/S) using BSA as a standard. Thirty µg of VLDL/LDL protein was labeled with either 0.6 MBq $^{131}$I or 0.7 MBq $^{125}$I in tubes coated with 10 µg iodogen (Invitrogen A/S, Taastrup, Denmark). The labeling efficiency was 15-32 %. The labeled lipoproteins were added 1 % BSA and passed over a PD-10 column to remove unbound $^1$I (GE Healtcare Life Sciences). Precipitation with 15 % trichloroacetic acid and extraction with chloroform:methanol (1:1) indicated that > 93-97 % of the radioactivity in the labeled VLDL/LDL preparations was attached to the protein moiety. To determine the plasma clearance of $^1$I-VLDL/LDL, male Ldlr$^{-/-}$ were fed Western type diet for 2 weeks and injected with $^{125}$I-VLDL/LDL from ApoM-Tg$^{Ldlr^{-/-}}$ mice and $^{131}$I-VLDL/LDL from Ldlr$^{-/-}$ mice (n = 3) or $^{131}$I-VLDL/LDL from ApoM-Tg$^{Ldlr^{-/-}}$ mice and $^{125}$I-VLDL/LDL from Ldlr$^{-/-}$ mice (n = 3). Each mouse received 2.3-3.2 x 10$^7$ cpm $^{125}$I and 3.2-4.3 x 10$^7$ cpm $^{131}$I in a total volume of 150 µl. Blood samples were taken 0, 5, 30, 120, 240, 480, and 1440 min after the injection. Aliquots of plasma samples were counted in a 1470 automatic gamma counter which allows for correction for $^{131}$I spillover into the $^{125}$I spectrum (PerkinElmer Danmark A/S, Skovlunde, Denmark) for 30 min. The experiment was repeated by injecting $^{125}$I-VLDL/LDL from ApoM-Tg$^{Ldlr^{-/-}}$ mice and $^{131}$I-VLDL/LDL from Ldlr$^{-/-}$ mice into eight Ldlr$^{-/-}$ and eight Lrp1$^{429n2}$Ldlr$^{-/-}$. Plasma samples taken at 5 and 360 min after injection were pooled and subjected to gel filtration followed by gamma counting of aliquots of gel filtration fraction.

To examine the re-distribution of apoM between lipoproteins in vivo, we injected human apoM-containing VLDL/LDL from ApoM-Tg$^{Ldlr^{-/-}}$ into three recipient Wt or Ldlr$^{-/-}$ mice (66 µg protein/mouse) and plasma containing HDL-associated human apoM from ApoM-Tg$^{Ldlr^{-/-}}$ into three recipient Wt or Ldlr$^{-/-}$ mice (110 ul/mouse). The putative distribution of human apoM from the injection lipoproteins among the plasma lipoproteins of the recipient mice was examined by plasma gel filtration and subsequent analyzes of human apoM by western blotting or ELISA using antibodies that do not cross-react with mouse apoM.

**Atherosclerosis.** Atherosclerotic lesions were evaluated by en face measurements of lesion areas or by morphometric analyses of histological cross sections from the aortic roots.

**Statistics.** Differences between groups were analyzed with $t$ tests. Welch’s correction for unequal variances was used whenever appropriate. Results are expressed as mean ± SEM.

**References**


2


Online Figure I. Standard curve for ELISA measurements of mouse plasma apoM. Ninety-six well plates were coated with polyclonal antibodies against mouse apoM and added diluted mouse plasma together with biotinylated recombinant mouse apoM. The absorbance on the y-axis reflects the amount of recombinant biotinylated apoM bound in the well. Note the absence of inhibition when adding plasma from ApoM$^{-/-}$ mice.
Online Figure II. Deficiency of LDL-receptors or impairment of LRPI increases apoM associated with HDL. ApoM in d < 1.063 g/mL (left) and d > 1.063 g/mL lipoproteins (right) in Wt, Ldlr<sup>-/-</sup>, ApoE<sup>-/-</sup>, and Lrp<sub>n2/n2</sub> mice. Plasma HDL and VLDL/LDL were separated after diluting 20 µL plasma samples to 200 µL and at the same time adjusting the solvent density to 1.063 g/mL. After ultracentrifugation, the top fraction was collected by aspiration. (A) ApoM was determined in the top and bottom fraction using the mouse apoM ELISA and expressed as percent of the concentration in a pool of plasma from Wt mice. Values are mean±SEM, n=8 in each group. *P<0.05 or **P<0.01 compared with Wt mice. (B) ApoB and apoE in the isolated fraction were visualized by western blotting. Note the increase of apoE in the HDL fraction of Ldlr<sup>-/-</sup> mice.
Online Figure III. Lowering of LDL-cholesterol in ApoB-Tg mice with an apoB-antisense oligonucleotide. (A) Plasma total cholesterol prior to (open symbols) and four days after an intraperitoneal injection of an apoB-antisense oligonucleotide (filled symbols). The number of mice in each group is indicated within the bars. (B) Plasma lipoprotein profiles were analyzed with gel filtration chromatography on a Superose 6 column using plasma pooled from ApoB-Tg mice prior to (open symbols) and four days after an intraperitoneal injection of an apoB-antisense oligonucleotide (filled symbols).
Online Figure IV. ApoM exchange rapidly between lipoprotein particles in vivo. Pooled plasma with human apoM-enriched HDL from ApoM-Tg and purified VLDL/LDL from ApoM-Tg/Ldlr-/- were injected intravenously into 3 recipient Wt mice and 3 recipient Ldlr-/- mice, respectively. (A) Gel filtration profiles of human apoM (ELISA measurements, left axis, open symbols) and cholesterol (right axis, filled symbols) in plasma of Wt recipient mice. (B) Gel filtration profiles of human apoM (ELISA measurements, left axis, open symbols) and cholesterol (right axis, filled symbols) in plasma of Ldlr-/- recipient mice.
Online Figure V. Plasma lipoprotein distribution of radioactivity upon injection of radio-iodinated VLDL/LDL into Ldlr⁻/⁻ mice. Ldlr⁻/⁻ mice (n=8) were co-injected with human apoM-rich ¹²⁵I-VLDL/LDL from ApoM-Tg⁺⁻Ldlr⁻/⁻ mice (filled symbols) and ¹³¹I-VLDL/LDL from Ldlr⁻/⁻ mice (open symbols). Pooled plasma samples collected 5 min and 6 h after the injection were subjected to gel filtration and radioactivity in each fraction was determined by γ-counting. The amount of ¹²⁵I and ¹³¹I in each gel filtration fraction is expressed as % of the total plasma counts at 5 min. Note that the amount of label remaining in the VLDL/LDL after 6 hours was larger for apoM-rich ¹²⁵I-VLDL/LDL from ApoM-Tg⁺⁻Ldlr⁻/⁻ than for apoM-poor ¹³¹I-VLDL/LDL from Ldlr⁻/⁻ mice.

C. Christoffersen et al
Online Figure VI. No effect of apoM overexpression on plasma lipoproteins in cholate/cholesterol-fed ApoM-Tg\textsuperscript{N} mice. Plasma lipoprotein profiles were analyzed with gel filtration chromatography on a Superose 6 column using plasma pools from 20-week old ApoM-Tg\textsuperscript{N} (filled symbols, n=14) and littermate Wt male mice (open symbols, n=9) that had been fed a cholate (0.5%) and cholesterol (1.25 %)-enriched diet from 4 weeks of age.

C. Christoffersen et al