Altered Metabolism of LDL in the Arterial Wall Precedes Atherosclerosis Regression

Emil D. Bartels,* Christina Christoffersen,* Marie W. Lindholm, Lars B. Nielsen

Rationale: Plasma cholesterol lowering is beneficial in patients with atherosclerosis. However, it is unknown how it affects entry and degradation of low-density lipoprotein (LDL) particles in the lesioned arterial wall.

Objective: We studied the effect of lipid-lowering therapy on LDL permeability and degradation of LDL particles in atherosclerotic aortas of mice by measuring the accumulation of iodinated LDL particles in the arterial wall.

Methods and Results: Cholesterol-fed, LDL receptor–deficient mice were treated with either an anti-Apob antisense oligonucleotide or a mismatch control antisense oligonucleotide once a week for 1 or 4 weeks before injection with preparations of iodinated LDL particles. The anti-Apob antisense oligonucleotide reduced plasma cholesterol by ≈90%. The aortic LDL permeability and degradation rates of newly entered LDL particles were reduced by ≈50% and ≈85% already after 1 week of treatment despite an unchanged pool size of aortic iodinated LDL particles. In contrast, the size, foam cell content, and aortic pool size of iodinated LDL particles of aortic atherosclerotic plaques were not reduced until after 4 weeks of treatment with the anti-Apob antisense oligonucleotide.

Conclusions: Improved endothelial barrier function toward the entry of plasma LDL particles and diminished aortic degradation of the newly entered LDL particles precede plaque regression. (Circ Res. 2015;117:933-942. DOI: 10.1161/CIRCRESAHA.115.307182.)

Key Words: atherosclerosis ■ lipoproteins ■ oligonucleotides, antisense ■ vascular permeability

Atherosclerosis is a leading cause of disability and death. Plasma low-density lipoprotein (LDL) particles promote the development of atherosclerosis when they cross the arterial endothelium. The LDL particles can be trapped in the intima-inner media and cause inflammation.2,3 Intimal inflammation attracts monocytes from the blood stream. They transform into macrophages that engulf and degrade the LDL particles, resulting in foam cell formation, that is, a pathological hallmark of atherosclerosis.2 Although plasma cholesterol lowering is beneficial in patients with atherosclerosis, it is unknown how it may affect the barrier function of the endothelium and subsequent metabolism of LDL particles in atherosclerotic lesions.

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Previous studies of the kinetics of LDL particles in the arterial intima of humans and experimental animal models have shown that although the endothelial LDL permeability is increased in atherosclerotic lesions,4,4 most LDL particles that enter the arterial wall leave (efflux) without contributing to the growth of the atherosclerotic lesion. However, the residence time of the newly entered particles is increased in lesioned versus nonlesioned arterial intima.4 This contributes to an increased pool size of LDL particles in the arterial intima and likely to increased susceptibility of the arterial LDL particles to aggregate and undergo other modifications within the arterial wall.7,9 Thus, promoting the growth of the lesion. The latter observation is one important foundation for the response to retention hypothesis of atherosclerosis.10

The rates of entry and degradation of LDL particles in the arterial wall have previously been studied in the aorta of rabbits. Both aortic LDL permeability and degradation of LDL particles are increased in atherosclerotic lesions.3,11-14 Hence, atherogenesis is accompanied by local changes in the arterial wall, which favors accumulation and degradation of plasma-derived LDL particles. However, it is not known whether these changes are irreversible and may react to, for example, cholesterol-lowering therapy.

Statin treatment reduces the size and changes the composition of established atherosclerotic lesions in humans.13 The reduction in the size of the atherosclerotic lesions is only modest although cardiovascular morbidity is markedly reduced.16

Morphological evaluations of atherosclerotic lesions, for

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example, in cholesterol-fed rabbits, suggest that lowering of plasma cholesterol (ie, after withholding cholesterol feeding) leads to plaque remodeling with formation of more collagen-rich atherosclerotic lesions after 6 to 12 months, suggesting that the long-term effect of plasma cholesterol lowering is a more stable plaque.17–20 This is also supported by data in mice, where genetic ablation of hepatic MTTP expression in LDL receptor–deficient mice (LDLr−/−) reduced plasma very low-density lipoprotein (VLDL) and LDL and led to more stable, less inflamed plaques.21 More recent studies where aortic segments from atherosclerotic donor mice with high plasma VLDL/LDL levels were transplanted into healthy recipient mice with low plasma VLDL/LDL levels suggest that atherosclerotic plaques are highly dynamic. Hence, transplantation of lesioned aortas into mice with nonatherogenic plasma lipid profiles reduces macrophage content in the transplanted aorta content within few days.22 Also, the phenotype of the remaining plaque macrophages shifts toward a less inflammatory M2 phenotype.23–26 Such cellular changes could possibly affect the rate of cellular degradation of LDL particles in the vessel wall and as such the regression of the atherosclerotic plaques. A deeper understanding of the mechanisms of atherosclerosis regression is an increasingly important area with the introduction and regulatory approval of potent lipid-lowering drugs, for example, proprotein convertase subtilisin kexin-9 inhibitors.27

We recently used an anti-Apob antisense oligonucleotide (ASO), which effectively reduces hepatic Apob mRNA expression by >90%.28 This compound allows elimination of the plasma VLDL/LDL exposure of a diseased arterial wall without the need for transplantation of aortic segments or change of diet. The aim of this study was to investigate the effect of lowering VLDL/LDL cholesterol on the aortic LDL permeability and the degradation rates of newly entered LDL particles in the arterial wall of mice with pre-existing atherosclerotic lesions.

Methods

An expanded Methods section is available in the Online Data Supplement.

Animals

Ldlr−/− mice were kept on a high-cholesterol diet for 16 to 19 weeks before treated weekly with anti-Apob ASO targeting Apob mRNA or control ASO (5 mg/kg per week, Santaris Pharma) for 1 or 4 weeks. The studies are outlined in Online Table I. All animal experiments were approved by the Danish Animal Experiments Inspectorate (Dyreforsoegstilsynet).

Isolation and Radioactive Labeling of LDL Particles Using Iodogen

Isolation and labeling of LDL particles with 125I or 131I were done essentially as previously described.29,30

LDL Permeability, Degradation, and Plasma Contamination

For estimation of arterial wall LDL permeability, mice (n=34) were injected with 125I-LDL in a tail vein 90 minutes before removal of the aorta. The same mice were also injected with 131I-LDL 47.5 minutes before removal of the aorta (n=17) to assess whether the accumulation of LDL particles coupled to radiolabeled iodine [125I-LDL increased linearly with time] or 5 minutes before removal of the aorta (n=17; to assess plasma contamination of the arterial tissue). Blood samples were drawn before injection and after 5, 45, 60, and 90 minutes.

For estimation of arterial wall LDL degradation, mice (n=31) were injected in a tail vein with 125I and 131I-TC/125I-LDL 23 to 25 hours before removal of aorta. Blood samples were drawn before injection and after 5 and 180 minutes and 23 to 25 hours.

Aliquots (10 µL) of plasma were precipitated with 15% trichloroacetic acid to remove free I and counted in a γ-counter, which allows the correction for 125I spillover into the 125I spectrum (1470 Automatic Counter, PerkinElmer Danmark A/S, Skovlunde, Denmark) for 10 minutes. The tissues were minced before precipitated with trichloroacetic acid and centrifuged. After centrifugation, the aortic radioactivity in the supernatant and precipitate was counted for 60 minutes.

Calculations

The calculations of the aortic LDL permeability and aortic degradation of radiolabeled LDL particles were corrected for the contamination of aortic tissue with plasma radioactivity.13,31

Tissue Samples

Before the last blood sample, the mice were anesthetized with an intraperitoneal injection of hypnorm/midazolam. Subsequently, tissue samples were obtained. The aorta was removed down to diaphragm, carefully cleaned from perivascular fat, and opened en face. The aortas were divided in an upper part (from the aortic root to the sixth rib) and a lower part (from the sixth rib to the diaphragm). Plaque surface areas were analyzed from photos taken with a Leica DFC290 digital camera mounted on a surgical microscope. The aortic intima-inner media was separated from the outer media and adventitia tissue using microforceps and a surgical microscope.

Biochemistry

Plasma cholesterol and triglycerides were determined with enzymatic methods (Chod-PAP: Roche, Denmark and GPO-Trinder; Sigma-Aldrich, Bromby, Denmark).

Size exclusion chromatography of mouse plasma pools (200 µL) was performed with a superose 6 column.32 The apo A1 and apoB levels in pooled gel filtration fractions corresponding to VLDL, LDL, high-density lipoprotein (HDL), or protein were determined with Western blot.33

Tissue Lipid Analysis

Aortic free and esterified cholesterol content and triglyceride content were measured with quantitative thin layer chromatography after extraction with chloroform/methanol.34

Histological Analyses

Hearts were immersion-fixed in paraformaldehyde (4%) and embedded in Tissue-Tek OCT (Sakura Finetek, Denmark). The proximal part of the heart containing the aortic root was sectioned in 10-µm sections and mounted on Superfrost Plus microscope slides (Thermo Scientific, Hvidovre, Denmark). Sections from the aortic root were stained with oil red O (ORO) to visualize lipids, collagen (Massons trichrome), macrophages (with anti-CD68 [catalog number: ab53444; Abcam, United Kingdom] or anti-CD163 antibodies [a kind gift from Dr Søren Mostrup; University of Aarhus]), scavenger receptors (anti-MSR [MSR1/scavenger receptor class A; catalog number: NBP-1-00092; Novus Biologicals], anti-CD36 [catalog number: NB-400-144; Novus Biologicals], and nitrotyrosine (antinitrotyrosine; catalog number: ab53444; Abcam, United Kingdom).
mRNA Expression
mRNA expression in total thoracic aorta and in laser-microdissected macrophages was determined with real time polymerase chain reaction using the ABI 7900 HT sequence detection system (Applied Biosystems, Foster City, CA).

Statistical Analysis
GraphPad Prism 4.0 (GraphPad Software Inc, San Diego) was used for statistical analyses. Two-group comparisons were done with Student’s t test or Mann–Whitney as stated in table and figure legends. P≤0.05 was considered significant.

Results
Anti-Apob ASO Effectively Reduces Plasma Cholesterol in Ldlr−/− Mice
To induce atherosclerosis in aorta, Ldlr−/− mice (n=98) were fed a cholesteroloriented diet for 16 weeks. Subsequently, while continuing the diet, the mice were randomly allocated to treatment with anti-Apob ASO or control ASO (5 mg/kg IP once weekly) for 1 or 4 weeks (Online Table I). Sixteen additional mice were fed the cholesteroloriented diet for 19 weeks before treated with ASO for 1 week, and 6 mice were fed the diet for 20 weeks without receiving any ASO treatment (Online Table I).

Anti-Apob ASO treatment lead to a sustained ≥90% reduction of total plasma cholesterol in cholesteroloriented Ldlr−/− mice when compared with control ASO treatment (Table 1). The reduction of plasma cholesterol was achieved within 2 to 3 days (Online Figure I). Plasma triglycerides were also reduced by the anti-Apob ASO (Table 1) consistent with the reduction in plasma VLDL. Accordingly, on size exclusion chromatography, plasma VLDL and LDL cholesterol and apoB were essentially eliminated in the cholesteroloriented anti-Apob ASO–treated Ldlr−/− mice both after 1 and 4 weeks of treatment (Figure 1A and 1D). The HDL cholesterol appeared slightly reduced by 35% after 1 week of anti-Apob ASO treatment (Online Figure IIA), whereas no changes in HDL cholesterol peak height was seen after 4 weeks of treatment (Online Figure IIB). Thus, the HDL/LDL ratio was markedly increased in anti-Apob ASO compared with control ASO–treated mice after both 1 and 4 weeks of treatment. Body weight was similar in anti-Apob ASO and control ASO–treated mice (Table 1).

Effect of Anti-Apob ASO on Plaque Size and Composition
After 1 week of treatment, the size of the atherosclerotic lesions in aorta, as judged either by en face analysis of the aorta or in histological cross section of the proximal aorta, was not affected by the anti-Apob ASO (Figure 2A and 2B). Also, there was no effect of anti-Apob ASO on plaque lipid content (as judged by ORO staining and chemical measurements of aortic cholesterol; Online Figure IIIA), collagen content (as judged by trichrome staining; Figure 2C and 2D), or the size of the necrotic core (Online Figure IIIC). In contrast, immunohistochemical staining of sections from the aortic root showed a significant reduction in the nitrotyrosine-stained area (an indirect marker of modification by reactive nitrogen species) after 1 week of treatment with the anti-Apob ASO (Figure 3G). Nevertheless, the amount of subendothelial foam cells (Figure 3E and 3F), the mRNA expression of inflammatory genes in the luminal subendothelial foam cells (Online Table III), and the expression of the scavenger receptors MSR, SR-BI, and CD36 were unchanged after 1 week of the cholesterol-lowering treatment (Online Figure IVA–IVC).

After 4 weeks of treatment, the morphology of the lesions was clearly affected. Hence, the size of the lesions and the ORO-stained plaque lipids were reduced, and the plaques contained relatively more collagen in the anti-Apob ASO–treated mice when compared with control ASO–treated mice (Figure 2E–2H). Remarkably, the anti-Apob ASO–treated mice lacked, or only had few, CD68-positive cells in the luminal parts of the atherosclerotic lesions, whereas these cells were abundant in the control ASO–treated mice (Figure 3A and 3B). This morphological difference also appeared in trichrome-stained sections where subendothelial foam cells were scarce in the anti-Apob ASO–treated mice (Figure 3C and 3D). On morphometry, the amount of subendothelial foam cells was reduced by P≤70% (P≤0.0001) in anti-Apob ASO compared with control ASO–treated mice (Figure 3E and 3F). The disappearance of the subluminal foam cells was accompanied by an 85% to 95% reduction in the mRNA expression of inflammatory genes in the aortic arch after 4 weeks of treatment (Table 2). Prototypical markers of M1 macrophages (Il-6, Inos, and Cd11c) and M2 macrophages (Cd206, Tgf-β, and Cd163) were reduced to the same extent. In contrast, the mRNA expression of Ccr7 and Tnf-α was unaffected by the anti-Apob ASO treatment (Table 2). Despite the reduction of foam cells, the aortic total cholesterol content (Online Figure IIIB) and the size of the necrotic core (Online Figure IIID) were not significantly reduced after 4 weeks of treatment. Immunohistochemical staining of sections from the aortic

Table 1. Plasma Lipids and Body Weight After Treatment With Anti-Apob or Control ASO

<table>
<thead>
<tr>
<th>Antisense treatment</th>
<th>1 wk of Treatment*</th>
<th>4 wk of Treatment†</th>
<th>No Treatment‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control ASO</td>
<td>16</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>Apob ASO</td>
<td>16</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Control ASO</td>
<td></td>
<td>26</td>
<td>6</td>
</tr>
<tr>
<td>Apob ASO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>24.7±0.5</td>
<td>24.7±0.3</td>
<td>N.D.</td>
</tr>
<tr>
<td>P-cholesterol, mmol/L</td>
<td>23.2±1.1</td>
<td>3.0±0.5§</td>
<td>21.7±1.4</td>
</tr>
<tr>
<td>P-triglyceride, mmol/L</td>
<td>1.94±0.54</td>
<td>0.35±0.12§</td>
<td>0.95±0.08</td>
</tr>
</tbody>
</table>

Data are mean±SEM. Two-group comparisons were done with Student’s t test. Further explanation is given in Online Table I. ASO indicates antisense oligonucleotide; and N.D., not determined.

*Studies 3 and 4.
†Studies 1, 2, and 5.
‡Study 6.
§P≤0.0001 compared with control ASO–treated mice.
root showed that the reduction in nitrotyrosine-stained area observed after 1 week was sustained after 4 weeks of treatment with the anti-Apob ASO (Figure 3H), whereas trends toward changes in the expression of the scavenger receptors MSR ($P=0.10$), CD36 ($P=0.06$), and SR-BI ($P=0.06$) did not reach statistical significance (Online Figure IVD–IVF).

Anti-Apob ASO Reduces Aortic LDL Permeability

The effect of plasma cholesterol reduction on the aortic LDL permeability was assessed by measuring the entry of LDL particle–bound *I in the intima-inner media of the aortic arch 45 and 90 minutes after an intravenous injection of *I-LDL before the removal of the aorta (studies 1 and 3).

The entry of *I in aortic intima-inner media increased from 45 minutes to 90 minutes after both 1 and 4 weeks of ASO treatment (Figure 4A and 4B). This indicates that the *I-LDL entry divided by the exposure time for ≤90 minutes primarily reflects the unidirectional flux of *I-LDL into the arterial wall and hence the aortic endothelial LDL permeability (in nL cm$^{-2}$ h$^{-1}$, where nL cm$^{-2}$ denotes nanoliters of plasma equivalents per square centimeter aortic surface area). The apparent larger entry of I-LDL from 45 to 90 minutes than from 0 to 45 minutes could reflect a lag in entry of I-LDL particularly in the anti-Apob ASO–treated mice. Notably, there was no difference in the rate of disappearance of *I-LDL from the plasma between anti-Apob ASO and control ASO–treated mice (Online Figure VC and VD).

The content of undegraded *I-LDL in the intima-inner media divided by the plasma concentration of *I-LDL at 24 hours provides an estimate of the pool size of undegraded LDL particles in the aortic intima-inner media. The pool size of undegraded LDL particles did not differ between anti-Apob ASO and control ASO–treated mice after 1 week (Figure 5A) but was reduced by ≈55% ($P=0.03$) in the anti-Apob ASO–treated mice after 4 weeks of treatment (Figure 5B).
The aortic LDL degradation was reduced from 1.7±0.21 nL cm⁻² h⁻¹ in control ASO–treated mice to 0.24±0.15 nL cm⁻² h⁻¹ in the anti-Apob ASO–treated mice (P=0.0006) already after 1 week of treatment. After 4 weeks of treatment with the anti-Apob ASO, LDL degradation was essentially eliminated from 0.73±0.07 nL cm⁻² h⁻¹ in control ASO–treated mice to 0.02±0.01 nL cm⁻² h⁻¹ in anti-Apob ASO–treated mice (P=0.0003; Figure 5C and 5D).

Effect of Anti-Apob ASO on Pinocytic Uptake of LDL Particles
To assess whether the sudden changes in LDL degradation on acute lipid-lowering treatment could be attributed to changes in macrophage pinocytosis, the nonreceptor-mediated uptake of LDL-sized particles by arterial wall macrophages was assessed by intravenous injection of pegylated fluorescent nanoparticles. After 1 week of anti-Apob ASO or control ASO treatment, there was no difference in the plaque accumulation of pegylated nanoparticles (Online Figure VI).

Discussion
Plasma LDL cholesterol fuels atherogenesis, and reduction of plasma LDL cholesterol improves the outcome of cardiovascular disease. However, little is known about the metabolism of LDL in the arterial wall during cholesterol-lowering treatment and atherosclerosis regression. The main results of this study are that lowering of plasma cholesterol leads to a pronounced reduction in the LDL entry in the arterial wall and in the propensity of the newly entered LDL particle to be degraded in the arterial wall, which precedes changes in the cellular composition and size of atherosclerotic lesions.

Using an anti-Apob ASO to lower plasma cholesterol, we achieved an essential elimination of plasma VLDL and LDL.
in Ldlr−/− mice with pre-existing atherosclerosis. Notably, this change occurred without changing the cholesterol-enriched diet. Within 4 weeks, atherosclerotic lesions become smaller and more collagen rich, suggesting plaque stabilization, which is in accordance with previous results from studies in cholesterol-fed rabbits.17–20 The sizes of the necrotic core and the aortic total cholesterol content were unchanged, possibly reflecting that the major pool of extracellular cholesterol is relatively resistant to efflux in the 4-week timespan studied, although the foam cell content was highly dynamic and disappeared already after 4 weeks of treatment. The discordance between the unchanged cholesterol levels in the thoracic aorta measured by thin layer chromatography and the reduced ORO staining in the aortic root of anti-Apob ASO–treated mice could, at least in part, be caused by changes in the hydrolysis/re-esterification cycle of cholesteryl esters in macrophages37 because unesterified cholesterol is not detected with ORO. The mass influx of LDL cholesterol is the product of the plasma LDL cholesterol concentration and the endothelial LDL permeability.38 Thus, any reduction of plasma VLDL and LDL is paralleled by a reduction in mass influx of cholesterol into the arterial wall. This study suggests that a reduction of the endothelial LDL permeability also contributes to an even further reduced mass influx

Figure 3. Effect of anti-Apob antisense oligonucleotide (ASO) treatment on macrophage foam cells in the aortic root. CD68-positive foam cells indicated with arrows in control ASO–treated mice (A) and anti-Apob ASO–treated mice (B) after 4 weeks of treatment. Trichrome staining of the aortic root after 4 weeks of treatment in control ASO–treated mice (C) and anti-Apob ASO–treated mice (D). The subendothelial foam cells are indicated with arrows. The cross-sectional area of foam cells was quantified in the mice after 1 week (E) and 4 (F) weeks of ASO treatment. Nitrotyrosine positive area in the aortic root after 1 week (G) and 4 weeks (H) of ASO treatment. Open boxes: control ASO–treated mice. Full boxes: anti-Apob ASO–treated mice. Data are presented as median with individual data points. Two-group comparisons were done with Mann–Whitney test.
of proatherogenic lipoproteins after cholesterol lowering. Hence, the aortic LDL permeability was reduced already 1 week after commencing anti-Apob ASO treatment, apparently without any changes in the general permeability toward albumin-sized plasma proteins. This finding suggests that the endothelial LDL permeability is specifically improved before the lesion size is reduced. Notably, the LDL permeability of the atherosclerotic murine aorta was comparable to what has previously been observed in rabbits, man, and pigs,5,6,11,39 supporting the validity of the present results in mice.

In addition to reducing the influx of *I-LDL, cholesterol lowering also lead to an 86% decline in fraction of newly entered *I-LDL that was degraded in the intima-inner media 1 week after commencement of anti-Apob ASO treatment. Notably, the reduction in the fraction of newly entered *I-LDL that was degraded within the arterial intima-inner media was more substantial than the reduction in influx of *I-LDL and occurred in the setting of an unchanged size of the pool of intact *I-LDL within the arterial wall. Because the macrophage is the primary cell type responsible for uptake and degradation of LDL particles,30 this result implies that the likelihood of a newly entered LDL particle to be taken up and degraded by arterial macrophage foam cells is dramatically reduced on lowering of plasma cholesterol. Remarkably, this was not because of the absence of macrophage foam cells in the arterial wall after 1 week. Instead, the result could reflect that cholesterol lowering lessens modifications of proteins by reactive nitrogen species within the arterial wall as suggested by decreased nitrotyrosine immune staining. Although the present immunohistochemical data on nitrosylation do not ascertain modifications of apoB per se, a decrease in LDL modifications within the arterial wall—and hence a decrease in cellular uptake—would be a plausible mechanism for our finding of lower degradation rates of newly entered LDL particles after anti-Apob ASO. Our results do not support the other major mechanistic possibility, namely, that the phenotype of the macrophages might have changed toward a cell type with less propensity to perform receptor-dependent or receptor-independent uptake of arterial LDL particles because we did not detect changes in the scavenger receptor protein expression, pinocytosis of nanoparticles, or even macrophage mRNA expression profiles.

After 4 weeks of treatment with anti-Apob ASO, the aortic degradation of *I-LDL was almost abolished, despite the fact that the reduction of the size of the arterial pool of *I-LDL was only 65%. This decline in degradation of newly entered LDL particles was paralleled by a massive reduction of subendothelial CD68-positive foam cells, which were abundant in the plaques of the control mice. The loss of macrophages was paralleled by a pronounced reduction in the mRNA content of macrophage-denominating inflammatory genes. Previous reports of gene expression in CD68-positive cells dissected from atherosclerotic plaques undergoing regression have suggested a prominent role for CCR7 in the process.24 We did not detect any difference in total aortic mRNA expression of CCR7 between mice treated 4 weeks with the anti-Apob and the control ASO. But because the number of foam cells was markedly reduced at this time point, we suspect that the per cell expression of CCR7 mRNA likely is increased on cholesterol lowering.

Despite similar changes in plaque composition, the changes in total aortic gene expression after 4 weeks of ASO...
treatment and the lack of changes in gene expression in laser-microdissected foam cell areas after 1 week of ASO treatment are in some contrast with previous studies of murine atherosclerosis regression. Hence, a selective induction of M2 markers and a downregulation of M1 markers have been seen already a few days after lipid-lowering interventions. Notably, in our model, we do not see an increase in plasma HDL accompanying the LDL-lowering treatment. Increases of HDL as seen in previous regression studies could affect both speed of regression, unloading of cellular cholesterol and the inflammatory phenotype of plaque macrophages.

In this study, the loss of foam cells was not accompanied by increased size of the necrotic core, implying that the foam cells may have left the arterial wall. Trogain et al demonstrated that foam cells indeed leave a transplanted arterial wall at an increased rate on exposure to a low plasma cholesterol environment. Hence, the disappearance of the foam cells as seen in this study might reflect egress to the blood.

The findings of an unchanged size of the necrotic core and similar levels of total aortic cholesterol content in anti-Apob ASO compared with control ASO–treated mice could imply that the majority of the cholesterol in atherosclerotic lesions is not easily mobilized in the 4-week timespan of this study despite marked changes in plasma lipid levels. This is in line with previous studies of atheroma regression in rhesus monkeys with established atherosclerotic disease, where the cholesterol content in coronary artery was significantly elevated despite regression of plaque size after >3 years on a low-cholesterol regression diet.

In conclusion, this study provides new insight into fundamental aspects of atherosclerosis regression. Hence, both improved endothelial barrier function toward plasma LDL particles and decreased propensity of newly entered plasma LDL particles to become degraded precedes morphological lesion regression with the loss of foam cells and markedly reduced lesion inflammation.

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Disclosures
M.W. Lindholm is an employee of Roche Innovation Center Copenhagen, Hoersholm, Denmark. The other authors report no conflicts.
pared with nonlesioned rabbit aorta.


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### What Is Known?
- Cholesterol lowering reduces the size of pre-existing atherosclerotic lesions only modestly.
- Nevertheless, cardiovascular morbidity is markedly reduced.

### What New Information Does This Article Contribute?
- Cholesterol lowering in mice rapidly reduced the entry into the aorta wall and subsequent cellular degradation of low-density lipoprotein (LDL) particles in mice with pre-existing atherosclerosis.
- Hence, reduced intra-aortic metabolism of LDL particles precedes morphological regression of pre-existing atherosclerosis on lowering of plasma cholesterol.

Atherosclerosis is a common cause of cardiovascular disease. Plasma LDL particles promote atherosclerosis by entering the arterial wall where the LDL particles can be modified and cause inflammation and foam cell formation. Although it is well understood how lowering of plasma cholesterol prevents development of atherosclerotic lesions, much less is known about molecular, cellular, or physiological effects on pre-existing atherosclerotic lesions. This study showed that aggressive plasma cholesterol–lowering treatment immediately improved the barrier function of the aortic endothelium toward plasma LDL particles in mice. Moreover, the degradation rate of newly entered LDL particles by cells in aorta was dramatically reduced. This profound change in the metabolism of a newly entered LDL particle by the arterial wall occurred already after 1 week of treatment and before any morphological signs of atherosclerosis regression. The data may imply that within arteries with pre-existing atherosclerosis, either modifications of LDL particles or the ability of foam cells to take up and degrade extracellular LDL particles are reduced when plasma LDL particles are lowered. Further delineation of the molecular and cellular mechanisms may point at new targets for treating pre-existing atherosclerosis at the level of the arterial wall.
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Detailed methods

Animals
Female (n = 92) and male (n = 30) Ldlr-/- (B6.129S7-Ldlrtm1Her/J, The Jackson Laboratory, Bar Harbor, USA) mice were housed under temperature-controlled conditions with free access to food and water at the Panum Institute, Copenhagen, Denmark. From 7-9 weeks of age, the mice were fed a high cholesterol diet (D01061402, Research Diet, New Brunswick, NJ, USA) to induce atherosclerosis. The mice were kept on the diet for 16-19 weeks before treated with Apob antisense oligonucleotide (anti-Apob ASO) or control ASO for one or four weeks. The studies are outlined in Supplementary Table 1. All animal experiments were approved by the Danish Animal Experiments Inspectorate (Dyreforsoegstilsynet).

ASO injections
To reduce plasma VLDL/LDL, mice were injected intraperitoneally with a 14-mer locked nucleic acid (LNA) antisense oligonucleotide targeting Apob mRNA (anti-Apob ASO, 5′-AGmCattggtatTmCA-3′, where the uppercase letters denote LNA monomers, lowercase letters denote DNA monomers, and superscript m indicates methylation, e.g. 5mC stands for LNA-5-methylcytidine). Control mice received a 13-mer LNA with four mismatching bases (control ASO, 5′-GmCatacgaaTCA-3′). The LNAs were prepared at Santaris Pharma, Hoersholm, Denmark, using standard procedure. The mice were randomly assigned to either treatment with anti-Apob ASO (5 mg/kg/week) or control ASO (5 mg/kg/week) once (1-week studies) or weekly in the 4-week studies (Supplementary Table 1).

Isolation and radioactive labeling of LDL
Isolation and labeling of LDL was done essentially as previously described. In brief, LDL was isolated by sequential ultracentrifugation from 25 ml plasma pooled from 15 human donors at density 1.019 g/ml and density 1.063 g/ml. The purified LDL fraction was dialyzed against 10 mM phosphate buffered saline (PBS) with 0.1 g/l Na2EDTA at 4 ºC for at least 16 hours and stored at 4 ºC for <24 hours before labeling. Protein concentrations were measured with the Pierce BCA protein assay kit (VWR-Bie & Berntsen A/S) using BSA as a standard. LDL (2 mg) was labeled with either 74 MBq 131I or 125I in tubes coated with 10 µg Iodogen (Invitrogen A/S, Taastrup, Denmark).

The labeling efficiency was 55% for 125I and 34% for 131I. BSA (1% in NaCl) was added to the labeled lipoproteins and the solution was passed over a PD-10 column equilibrated with PBS to remove unbound *I (GE Healthcare Life Sciences). Precipitation with 15% trichloroacetic acid and extraction with chloroform:methanol (1:1) indicated that 95-97% of the radioactivity in the labeled LDL preparations was attached to the protein moiety. The labeled lipoproteins were stored at 4 ºC and used for IV injection within 36 hours.

For the studies of LDL-degradation, mice were injected with LDL that was double-labeled with 125I (as described above) and 131I-TC. Upon degradation of the double-labeled LDL, the 131I-TC moiety is trapped inside cells whereas the 125I diffuses out of the cells. For 131I-TC labeling of LDL, TC (50 nmol) was labeled with 185 MBq 131I in tubes coated with 10 µg Iodogen (Invitrogen). Then, the 131I-TC was transferred to a vial containing 10 µL NaHSO3 (0.1 mol/L) and 5 µL NaI (0.1 mol/L) and activated by the addition of 20 µL cyanuric chloride (2.5 mmol/L in acetone) followed by 5 µL NaOH (0.02 mol/L) and 10 µL acetic acid (0.015 mol/L) before coupled to 5 mg human 125I-LDL at pH 9–10. To remove unbound 131I and 131I-TC, the mixture was passed over a PD-10 column (Amersham Biosciences). More than 93% of 125I and 89% of 131I was precipitated with 15% trichloroacetic acid in preparations of 131I-TC/125I-LDL.

LDL permeability, LDL-Degradation and plasma contamination
To determine aortic LDL permeability mice were injected with 150-160 µl (1.4-15 × 10⁶ cpm) 125I-LDL in a tail vein 90 minutes prior to removal of the aorta. The same mice were also injected with 150-160 µl (2.8-14 × 10⁶ cpm) 131I-LDL 47½ minutes prior to removal of the aorta (n = 17) to assess whether the accumulation of 131I-LDL increased linearly with time, or 5 minutes prior to removal of the aorta (n = 17) to assess plasma contamination of the arterial tissue. Blood samples (50-100 µl) were drawn before injection (retro-orbital), after 5 minutes (retro-orbital), 45 minutes (tail vein), 60 minutes (tail vein), and 90 minutes
(retro-orbital). Before the last blood sample, the mice were anaesthetized with an intraperitoneal injection of hypnorm/midazolam. The mice were sacrificed in the anesthesia and tissue samples were then obtained.

To determine aortic LDL degradation mice (n = 31) were injected in a tail vein with 200 µl (2.0-2.2 ×10^6 cpm of ^125^I and 4.5-5.2 ×10^5 cpm of ^131^I) ^131^I-TC/ ^125^I-LDL 23-25 hours before removal of aorta. Blood samples (50-100 µl) were drawn before injection (retro-orbital), after 5 minutes (tail vein), 180 minutes (tail vein) and 23-25 hours (retro-orbital). Before the last blood sample the mice were anaesthetized with an intraperitoneal injection of hypnorm/midazolam. The mice were sacrificed in the anesthesia and tissue samples were then obtained. The I-LDL injections maximally increased plasma cholesterol by ~2 ‰. Aliquots (10 µl) of plasma were precipitated with 15% TCA to remove free *I and counted in a gamma counter which allows correction for ^131^I spillover into the ^125^I spectrum (1470 Automatic Counter, PerkinElmer Danmark A/S, Skovlunde, Denmark) for 10 minutes. The aortic intima-inner media was separated from the outer media and adventitia tissue using micro forceps and a surgical microscope. The tissue was minced with scissors before precipitated with TCA and centrifuged. After centrifugation, the aortic radioactivity in the supernatant and precipitate was counted for 60 min (1470 automatic gamma, PerkinElmer Danmark A/S, Skovlunde, Denmark).

### Calculations

The calculations of the aortic LDL permeability and aortic degradation of radiolabeled LDL were corrected for the amount of radioactivity sticking to the tissue samples at the time of removal from the experimental animals (plasma contamination). Plasma contamination was estimated in a subgroup of mice (n = 17) as the TCA-precipitable radioactivity in aortic tissue of ^131^I-LDL injected 5 minutes prior the removal of the aorta divided by the area of the aortic segment and the TCA-preciptable ^131^I radioactivity in plasma at the end of the experiment. At this time point only a minute part of the label will have penetrated the vessel wall, and the measured ^131^I-radioactivity can therefore be assumed to be a measure of plasma adhering to the luminal side of the vessel.38 The plasma contamination was 26.2 ± 3.6 nl·cm⁻² (where nl·cm⁻² denotes nanoliters of plasma equivalents per square centimeter aortic surface area) and was not different between experimental groups, and therefore the mean of all determinations was used in subsequent calculations. The overall results of the study remained the same, regardless of whether the results were corrected for contamination or not. The magnitude of plasma contamination in the present study was comparable with earlier studies in rabbits.5,7

The aortic LDL permeability was calculated as: (TCA-perceptible radioactivity in the intima-inner media per cm² corrected for contamination) divided by the mean exposure to radioactivity during the experiment, as previously described.8

The degradation-product was calculated as the difference between accumulated ^131^I (representing both intact and degraded LDL) and ^125^I (representing intact LDL) in the intima/inner media. Calculation of accumulated LDL was done with the formula: (TCA precipitable radioactivity per cm² intima/inner media minus the plasma contamination times plasma radioactivity at 1440 minutes) divided by average exposure to radiolabeled LDL.5

The poolsize of undegraded LDL was calculated as the accumulated amount of ^125^I (representing intact LDL) in the intima/inner media corrected for contamination divided by the plasma concentration of ^125^I 24 hours after injection under the assumption, that equilibrium between plasma and aorta has been reached at this time point.9

### Evans blue permeability

To determine the general entry of plasma proteins into the aortic vessel wall, cholesterol-fed mice treated with anti-Apob ASO (n=6) or control-ASO (n=6) for one weeks were injected intravenously with 100 µl 1 % Evans Blue in PBS. Thirty minutes after the injection, the mice were anaestesized, perfused with saline, and the aortas and hearts were removed for later analysis. Evans Blue was extracted with formamide and quantified as described by Phinikaridou et al10, or visualized en face in the thoracic aorta using the 700 nm filter on an Odyssey Fc® instrument (Licor-biotechnology, Cambridge, United Kingdom).

### Pinocytosis

2
To determine, in vivo, the non-receptor mediated uptake of LDL-sized particles in macrophages in atherosclerotic plaques cholesterol-fed mice treated with anti-Apob ASO (n=8) or control-ASO (n=8) for one week were injected intravenously with 100 µl Angio SPARK 680 nano particles (Perkin Elmer, Skovlunde, Denmark). Twenty four hours after the injection, the mice were perfused with 0.9 % saline, and the aortas and hearts were removed for later analysis. Uptake of Angio SPARK 680 nano particles in atherosclerotic plaques was visualized in 3 cross sections of the aortic root per mouse (6 mice per group) with a Zeiss Axio Scan.Z1 microscope and quantified in the Zeiss Zen Blue software (Carl Zeiss Microscopy GmbH, Jena, Germany). Total fluorescent area of the aortas was measured en face using the 700 nm filter on an Odyssey Fe instrument (Licor-biotechnology, Cambridge, United Kingdom).

**Tissue samples**

The aorta was removed down to the diaphragm, carefully cleaned from peri-vascular fat and opened en face. The aortas were divided in an upper part (from the aortic root to the sixth rib) and a lower part (from the sixth rib to the diaphragm). Plaque surface areas were analyzed from photos taken with a Leica DFC290 digital camera mounted on a surgical microscope before the aortic segments were processed for estimation of radioactivity as described above.

**Biochemistry**

Blood samples were drawn from the retro-orbital plexus in pre-cooled Na2-EDTA tubes for determination of plasma lipids after 1, 3, and 4 weeks of treatment. Plasma cholesterol and triglycerides were determined with enzymatic methods (Chod-PAP, Roche, Denmark and GPO-Trinder, Sigma-Aldrich, Brondby, Denmark, respectively).

Size exclusion chromatography of mouse plasma (pools of 200 µl made from 7-10 mice/group) was performed at 20–24 °C using PBS-EDTA and a Superose 6 10/300 GL fast protein liquid chromatography (FPLC) column (GE Healthcare). The flow rate was 0.2–0.4 ml/min. Fractions of ∼250 µl were collected and stored at −20 °C. Pooled gel filtration fractions corresponding to VLDL, LDL, HDL, or protein were used for western blot with antibodies against apoA-I and apoB. In brief, aliquots of pooled fractions gel filtration fractions were separated on 12 % SDS-PAGE gels and western blotting visualizing mouse apoA-I using rabbit anti-mouse apoA-I antibody (1:5000) (Nordic Biosite Aps, Copenhagen, Denmark) and mouse apoB using rabbit anti-mouse apoB48/100 antibody (1:1000) (Nordic Biosite Aps, Copenhagen, Denmark) was performed.

**Lipid analysis**

Aortic lipids were extracted in 1.25 mL methanol and 2.5 mL chloroform and after evaporation of organic solvents, redissolved in 50 µL toluol. Levels of free and esterified cholesterol content were measured with quantitative thin layer chromatography. All samples were analyzed in duplicate on separate TLC plates. To account for differences in aorta size data were normalized by the area of the aortic arch (in µm2) as previously described.

**Histological analyses**

To visualize neutral lipids, sections from the aortic root were rinsed in distilled water and immersed in Oil red O for 15 minutes (cat.no.O-0625, Sigma Aldrich, Copenhagen, Denmark) (0.5 g dissolved in 100 ml 99 % isopropanol, filtered and diluted 4:10 in distilled water), rinsed in 60 % isopropanol and tap water before counterstained with Meyers haematoxylin and mounted with fluorescence mounting medium (Dako, Glostrup, Denmark). To visualize collagen in plaques, sections from the aortic root were rinsed in distilled water and stained with Masson Trichrome Stain (Sigma Aldrich, Copenhagen, Denmark). The sections were dehydrated in alcohol and xylene before mounted with pertex. For immunohistochemical staining, the sections were rinsed in distilled water, incubated in 1% H2O2 for 3 minutes, with protease K for 5 minutes before blocking with 5% goat serum in TBS for 30 minutes. The sections were incubated with primary antibody in 5% goat serum overnight (CD68 1: 50 (ab53444, Abcam, UK), CD163 1:100 (a kind gift from Dr. Søren Mostrup, University of Aarhus), SRBI 1:1000 (NB400-104, Novus Biologicals), CD36 1:250 (NB400-144, Novus Biologicals), MSR 1:1000 (NBP-1-00092, Novus...
Biologicals) and nitrotyrosine 1:100, (cat.no. ab53444, Abcam, UK). The sections were subsequently washed and developed with either a biotinylated goat-anti rat antibody and Vectorstain ABC-kit (CD68) (cat.no. PK-4005, VWR, Roedovre, Denmark) or Envision™ (CD163, CD36, MSR, nitrotyrosine and SRBI) (Dako, Glostrup, Denmark). Counterstaining was done with Meyers haematoxylin followed by dehydration in alcohol and xylene and mounting with pertex. The Visiopharm (Hoersholm, Denmark) and IM50 (Leica, Denmark) software packages were used for quantitative image analysis.

**mRNA expression**

From mice treated 4 weeks with the anti-Apob or control ASO mRNA was extracted from the thoracic aorta with Trizol (Invitrogen, Taastrup, Denmark), quantified spectrophotometrically, and quality assured with capillary electrophoresis (2100 bioanalyzer, Agilent Technologies). cDNA was synthesized with M-MULV (Roche) and real-time PCR analyses was performed using Fast SYBR Green Master Mix and the ABI 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). Reaction volumes were 20 μl using template cDNA synthesized from 10 ng total RNA. All analyses were done in duplicate, and gene expression data were normalized with the amount of 18S RNA in the same cDNA preparation.

From mice treated for 1 week with the anti-Apob or control ASO, subendothelial macrophage rich regions were isolated from 10μm sections using a Zeiss Laser Capture Microdissecting system (LCM). The mRNA was extracted with RNAqueous-Micro kit (Ambion). Real-time PCR analyses was performed with Fast SYBR Green Master Mix and the ABI 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). Reaction volumes were 20 μl using template cDNA synthesized from 10 ng total RNA using the SuperScript III, First Strand Synthesis (Cat.nr 18080-400, Invitrogen). All analyses were done in duplicate, and gene expression data were normalized with the amount of 18S RNA in the same cDNA preparation.

Supplementary Table 2 contains primer sequences.

**Statistics**

GraphPad Prism 4.0 (GraphPad Software Inc., San Diego) was used for statistical analyses. Two-group comparisons were done with Student's *t*-test or Mann-Whitney's test as stated in table and figure legends. *P* ≤ 0.05 was considered significant.
Online Figure I. Effect of a single injection of two different doses of anti-AApob ASO on total plasma cholesterol in chow-fed LDLr⁻/⁻ mice. Open circles: 5 mg/kg, closed circles: 20 mg/kg. Data are presented as mean ± SD.
Online Figure II. Cholesterol profiles of plasma pools from 9-10 mice/group separated with size exclusion chromatography. The inserts are showing the HDL cholesterol peaks, (A) after one week of treatment and (B) after 4 weeks of treatment with the anti-Apob or control ASO.
Online Figure III. Aortic cholesterol content (A) & (B) and size of necrotic plaque area at the level of the aortic root (C) & (D) after control ASO and anti-Apob ASO treatment for 1 week (A) & (C) or 4 weeks (B) & (D) of treatment. Open squares represent control ASO treated mice, full squares represent anti-Apob ASO treated mice. Values are individual data point with medians.
Online Figure IV. Immunostainings of macrophage receptors in sections of the aortic root after 1 week (A)-(C) and 4 weeks (D)-(F) of treatment with control ASO or anti-Apob ASO, n=4 mice per group. Open boxes: control ASO treated mice; full boxes: anti-Apob ASO treated mice. Values are individual data point and medians. P-values obtained with Mann-Whitney's test.
Online Figure V. Plasma decay of injected radiolabeled LDL after control ASO and *anti*-Apob ASO treatment for 1 week (C-F) or 4 weeks (A-B & G-H). Decay for up to 90 minutes was measured in the studies of aortic LDL permeability (A-D) and for up to 24 hours in the studies of aortic LDL degradation (E-H). Squares represent control ASO treated mice and triangles *anti*-Apob ASO treated mice. Values are shown as median with interquartile ranges.
**Online Figure VI.** One week of *anti-Apob* ASO treatment does not affect the in vivo uptake of pegylated nanoparticles in plaque macrophages. (A) Pinocytotic uptake of fluorescent nanoparticles in thoracic aorta judged by en face measurement of fluorescence 24 hours after injection of Angio SPARK 680. Data are individual points with medians. Open circles: saline injected, non-treated mice. Open squares: control ASO treated mice injected with Angio SPARK 680. Closed squares: *anti-Apob* ASO treated mice injected with Angio SPARK 680. (B) Aortas from mice injected with Angio SPARK 680 (aortas 1-3) or saline (aorta 4). Angio SPARK 680 depicted with red color, aortic autofluorescence in grey. (C) Pinocytotic uptake of fluorescent nanoparticles visualized in cross sections of the aortic root. Data are presented as individual points with medians. Open squares: control ASO treated mice. Closed squares: *anti-Apob* ASO treated mice. (D) Picture of the aortic root from a mouse injected with Angio SPARK 680 24 hours before sacrifice. 1: Aortic lumen; 2: subendothelial macrophage rich area; 3: necrotic core; 4: media and adventitia. Angio SPARK 680 depicted with red color and cell nuclei with blue.
### Online Table 1a – Study design 1 week studies

<table>
<thead>
<tr>
<th>Study</th>
<th>3</th>
<th>4</th>
<th>7</th>
<th>8</th>
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</thead>
</table>
| **ASO treatment**               | control | anti-
ApoB | control | anti-
ApoB | control | anti-
ApoB | control | anti-
ApoB |
| Number of mice                  | 8      | 8     | 6     | 6     | 8      | 8     |
| Weeks on diet before ASO       | 16     | 16    | 19    | 19    | 16     | 16    |
| Weeks of ASO treatment         | 1      | 1     | 1     | 1     | 1      | 1     |
| **Measurements**               |        |       |       |       |        |       |
| Plasma biochemistry             | +      | +     | +     | +     | +      | +     |
| Surface plaque area             | +      | +     | +     | +     | +      | +     |
| Cross sectional plaque area    | +      | +     | +     | +     | +      | +     |
| Aortic cholesterol content     | +      | +     | +     | +     |        |       |
| LDL-permeability               | +      | +     |       |       | +      | +     |
| LDL-degradation                |        |       |       |       |        |       |
| Gene-expression in aorta        | +*     | +*    |       |       |        |       |
| CD36, MSR, SR-BI & nitrotyrosine IHC |       |       |       |       |        |       |
| Evans Blue permeability        | +      | +     |       |       | +      | +     |
| Angiospark 680 accumulation    |        |       |       |       |        |       |

*\(n=4\)
**Online Table Ib** – Study design 4 week studies

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<td>anti-</td>
<td>control</td>
<td>anti-</td>
</tr>
<tr>
<td></td>
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<td>2</td>
<td>5</td>
<td>6</td>
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<tr>
<td><strong>Number of mice</strong></td>
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<tr>
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**Measurements**

- **Plasma biochemistry**
  + + + + + + + +
- **Surface plaque area**
  + + + + + + + *
- **Cross sectional plaque area**
  +* +* + +
- **Aortic cholesterol content**
  +
- **LDL-permeability**
  + +
- **LDL-degradation**
  +
- **Gene-expression in aorta**
  + +
- **CD36, MSR, SR-BI & nitrotyrosine IHC**
  +** +**

* Data not shown, **n=4*
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<th>Gene</th>
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Online Table III. Expression of inflammatory genes in subendothelial macrophages purified with LCM from thoracic aorta after treatment with *anti-Apob* or control ASO for 1 week.

<table>
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<th><em>Anti-Apob</em> ASO treated mice (n=6)</th>
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<tr>
<td>MCP-1</td>
<td>0.88 (0.52-1.95)</td>
<td>0.95 (0.67-2.26)</td>
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<tr>
<td>CD206</td>
<td>1.02 (0.22-1.58)</td>
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<td>SR-BI</td>
<td>0.46 (0.26-0.75)</td>
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<td>CD36</td>
<td>0.94 (0.56-1.01)</td>
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<td>CD11c</td>
<td>2.36 (0.33-2.52)</td>
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<td>CCR7</td>
<td>0.006 (0.002-0.009)</td>
<td>0.080 (0.002-0.399)</td>
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</table>

The relative mRNA expression level of macrophage related genes subendothelial macrophage rich regions (isolated with LCM) in the thoracic aorta normalized to the 18S expression in the same cDNA preparation and PCR-run (expressed in arbitrary units). No differences in 18S mRNA expression or RNA quality were observed between groups. All values are medians with interquartile ranges. Two-group comparisons were done with Mann-Whitney test.
Online References


