Disturbed Laminar Blood Flow Vastly Augments Lipoprotein Retention in the Artery Wall
A Key Mechanism Distinguishing Susceptible From Resistant Sites

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Objective—Atherosclerosis develops initially at branch points and in areas of high-vessel curvature. Moreover, experiments in hypercholesterolemic mice have shown that the introduction of disturbed flow in straight, atherosclerosis-resistant arterial segments turns them highly atherosclerosis susceptible. Several biomechanical mechanisms have been proposed, but none has been demonstrated. In the present study, we examined whether a causal link exists between disturbed laminar flow and the ability of the arterial wall to retain lipoproteins.

Approach and Results—Lipoprotein retention was detected at natural predilection sites of the murine thoracic aorta 18 hours after infusion of fluorescently labeled low-density lipoprotein. To test for causality between blood flow and the ability of these areas to retain lipoproteins, we manipulated blood flow in the straight segment of the common carotid artery using a constrictive collar. Disturbed laminar flow did not affect low-density lipoprotein influx, but increased the ability of the artery wall to bind low-density lipoprotein. Concordantly, disturbed laminar flow led to differential expression of genes associated with phenotypic modulation of vascular smooth muscle cells, increased expression of proteoglycan core proteins associated with lipoprotein retention, and of enzymes responsible for chondroitin sulfate-glycosaminoglycan synthesis and sulfation.

Conclusions—Blood flow regulates genes associated with vascular smooth muscle cell phenotypic modulation, as well as the expression and post-translational modification of lipoprotein-binding proteoglycan core proteins, and the introduction of disturbed laminar flow vastly augments the ability of a previously resistant, straight arterial segment to retain lipoproteins. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.305874.)

Key Words: aorta ■ atherosclerosis ■ glycosaminoglycans ■ lipoproteins ■ mice
Other sets of data, however, speak to the importance of lipoprotein retention in initiating lesion development. In vitro binding studies have established proteoglycans as the main lipoprotein-binding component of the vessel wall because negatively charged sulfated glycosaminoglycan moieties—especially chondroitin sulfate-glycosaminoglycans—bind electrostatically to positively charged residue stretches of apolipoprotein B
sub, apoB
sub, and apoB
sub. The response-to-retention theory states that the binding of atherogenic lipoproteins to these constituents in the intimal layer of the artery wall is the key initiating event in lesion development, and that other atherogenic processes are a consequence thereof. It also predicts that altered shear stress affects atherosclerosis by regulating the ability of the intima matrix to bind lipoproteins, but this chain of causation has yet to be directly demonstrated.

In the present study, we show that such a causal link exists in mice. Using experimental manipulation of blood flow in carotid arteries, we find that disturbed laminar flow directly augments the ability of the artery wall to retain lipoproteins.

Materials and Methods
Materials and Methods are available in the online only Data Supplement.

Results
LDL Is Retained at Atherosclerosis Predilection Sites
To study physiological retention of LDL in vivo, Atto 680–labeled human LDL was infused into chow-fed, normocholesterolemic C57BL/6 mice, which were perfusion-fixed 18 hours later. At this time point, infused human LDL was completely cleared from the circulation (Figure 1A), and any LDL detected in the artery wall was interpreted as immobilized, ie, retained. Thoracic aortas were isolated, opened longitudinally, and scanned using infrared imaging. LDL was retained along the inner curvature of the aortic arch and at branch points (Figure 1B), sites known to be susceptible to atherosclerosis in both mice and humans.

To determine the exact location of retained LDL within the artery wall, the same experiment was performed with Atto 565–labeled human LDL, and longitudinal cryosections of the aortic arch were analyzed by fluorescence microscopy. In the inner curvature of the aortic arch, LDL was located mainly in the medial layer between elastic lamellae among vascular smooth muscle cells (VSMCs) and in the intimal layer (Figure 1C). No LDL was detected in the outer curvature (Figure 1D), consistent with the observations when using Atto 680–labeled LDL.

Figure 1. Low-density lipoprotein (LDL) is retained in atherosclerosis-prone areas of normal mice. A. Infused human LDL is cleared from the circulation within 18 hours. The graph shows levels of human apoB
sub in murine plasma in noninfused (control) mice (n=3) and 1 (n=4) and 18 (n=5) hours after LDL infusion as assayed with a human-specific apoB ELISA. Lines on graph indicate medians. B. Longitudinally opened thoracic aorta showing retention of Atto 680–labeled LDL (in black) in atherosclerosis-prone areas in the inner aortic curvature and at branch points 18 hours post infusion. C. Microscopic analysis of the intimal and medial location of Atto 565–labeled LDL (in red) in the inner curvature of the aortic arch. D. No Atto 565–labeled LDL is retained in the outer curvature of the aortic arch. Asterisks indicate positions of artery lumen. Arrows point to internal elastic lamella. Similar images to those shown in B–D were obtained from a total of four or three mice in each experiment. Scale bars, 50 µm.

Introduction of Disturbed Laminar Blood Flow in the Straight Segment of Murine Carotid Arteries Vasily Augments LDL Retention
The artery wall forming the inner curvature of the aortic arch and branch sites is exposed to disturbed laminar flow, and we thus speculated whether the disturbed laminar flow was sufficient to transform vascular sites into LDL-retaining regions. To test this hypothesis, we manipulated blood flow in the right CCA using the perivascular collar technique described by von der Thüsen et al. Under normal conditions, the straight segment of the CCA is exposed to uniform laminar flow and is resistant to atherosclerosis. However, implanting a mildly constricitive perivascular collar has been shown to evoke disturbed laminar flow in the segment proximal to the collar characterized by low wall shear stress and cyclic circumferential stretching because of periodic increases in transmural pressure. These characteristics are comparable with those of natural predilection sites such as the inner curvature of the aortic arch and cause rapid plaque development in the presence of hypercholesterolemia.

To validate our adaption of this technique, we first reproduced the results of von der Thüsen et al in apolipoprotein E (apoE)–deficient mice. After 6 weeks on a Western-type diet, severe atherosclerosis had developed proximal to constrictive collars, whereas lesion development was negligible proximal to nonconstrictive collars (Figure 2A–2C).

We then applied constrictive and nonconstrictive collars to normocholesterolemic C57BL/6 mice. After a period of 2 weeks, both groups were infused with Atto 680–labeled LDL and perfusion-fixed 18 hours later. Scans of intact CCAs showed that infused LDL was retained proximal to constrictive
collars, but not proximal to nonconstrictive collars (Figure 3A and 3B). We conclude that disturbed laminar flow is a sufficient cause for the generation of lipoprotein-retaining vascular sites in normal mice.

### LDL and Not Albumin or apoA1 Contaminants Provide the Fluorescence-Retention Signal

The purity of Atto 565 NHS ester–labeled LDL was assessed by gel filtration chromatography, and trace contamination of albumin (<1%) was detected (data not shown). To exclude that the assayed fluorescence signal was caused by albumin or other potential contaminants in the LDL preparations, which would also be conjugated with the fluorochrome, we selectively removed LDL after Atto 565 NHS ester conjugation with beads cross-linked to rabbit antihuman apoB IgG or, as a negative control, beads cross-linked to the immunoglobulin fraction from nonimmunized rabbits. Immunoprecipitation with anti-apoB antibodies resulted in efficient and selective removal of apoB<sub>100</sub> from the sample, while preserving the contaminants albumin and apoA1 (Figure 4A). Preparations were then infused into mice 2 weeks after collar insertion and the mice were fixated 18 hours post infuion as in previous experiments. Although the preparation incubated with control beads resulted in strong Atto 565 fluorescence from the CCA segment exposed to disturbed laminar flow, removal of LDL from the preparation fully abolished this, thereby validating that the signal used for quantification represents LDL and not contaminants (Figure 4B).

### Disturbed Laminar Flow Increases Binding of LDL in the CCA

Accumulation of infused LDL at sites exposed to disturbed laminar flow could be caused either by an increase in lipoprotein influx (an increase in endothelial permeability or by flow itself, which could simply increase delivery of LDL into the artery wall) or a decrease in lipoprotein efflux by an increase in the ability of the artery wall to bind and to retain LDL. To address this question, mice with constrictive or nonconstrictive collars implanted for 2 weeks were perfusion-fixated 1 hour after infusion of Atto 565–labeled LDL. At this time point, infused LDL is still present in the circulation (Figure 1A). LDL detected in the arteries of these mice thus represents both mobile and immobile LDL and assays a combination of influx

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**Figure 2.** Disturbed laminar flow makes the straight segment of the common carotid artery susceptible to atherosclerosis in apo-e-deficient mice. **A** and **B**, Representative images of cross sections proximal to constrictive and nonconstrictive collars, respectively, after 6 weeks on Western-type diet. Arrows point to internal elastic lamella. Scale bars, 100 μm. **C**, Atherosclerosis lesion size expressed as the average intimal area in the sections collected from the 2-mm segment proximal to constrictive (n=3) and nonconstrictive (n=5) collars. *P*<0.05 (Mann–Whitney test). Lines on graph indicate medians.

**Figure 3.** Introduction of disturbed laminar blood flow in the straight segment of murine carotid arteries vastly augments low-density lipoprotein (LDL) retention. **A**, Principle of Atto 680–labeled LDL quantification. **Top** and **bottom** row, Representative examples of common carotid arteries exposed to constrictive and nonconstrictive collars, respectively. Black represents retained LDL. Red color represents area with pixel values above background used for quantification. Dashed line shows position of the proximal collar edge. Scale bar, 1 mm. Vertical arrows indicate direction of blood flow. **B**, Atto 680–labeled LDL is retained in the 1-mm segment proximal to constrictive (n=7), but not to nonconstrictive collars (n=5). *P*<0.05 (Mann–Whitney test). Lines on graph indicate medians.

**Figure 4.** Low-density lipoprotein (LDL) and not albumin or apoA1 contaminants provide the fluorescence-retention signal. **A**, SDS-PAGE gels (3%–8% and 12%) showing specific removal of LDL (apoB<sub>100</sub> [515 kDa] indicated by red arrow), while preserving contaminants (albumin [67 kDa] and apoAI [28.1 kDa] indicated by black and gray arrows, respectively). Molar weight is shown in kDa. **B**, Retention of Atto-565–labeled LDL (n=5) and not of Atto-565–labeled albumin and apoA1 contaminants (n=5) provides a fluorescence signal proximal to constrictive collars applied 2 weeks in advance and assayed 18 hours post infusion. *P*<0.05 (Mann–Whitney test). Lines on graph indicate medians.
and initial binding. For comparison, the same experiment was set up with mice perfusion-fixated 18 hours post LDL infusion, thereby assaying retention as in previous experiments (Figure 5A). Serial cross sections from the 500-µm segment proximal to the collar were systematically sampled and analyzed to quantify the amount of LDL as shown in Figure 5B to 5G. Interestingly, there was no detectable difference between the amount of LDL proximal to constrictive and nonconstrictive collars 1 hour after LDL infusion. In contrast, 18 hours after infusion, LDL was retained proximal to constrictive collars, but not to nonconstrictive collars (thereby replicating the results shown in Figure 3 with an alternative quantification technique; Figure 5H–5N). As for the inner curvature of the aortic arch (Figure 1C), LDL was located both in the medial and in the intimal layers (Figure 5D and 5L).

In conclusion, flow-induced retention in the CCA was not mediated through an increased influx by either increased endothelial permeability or flow-mediated transfer without vascular changes, but by changes in the ability of the vascular wall to bind, ie, or to retain LDL (Figure 5O).

**Disturbed Laminar Flow Regulates Predefined Gene Sets Associated With Lipoprotein Retention**

As LDL was located among VSMCs in the medial layer of the artery wall, we speculated that the disturbed laminar flow might have affected VSMC phenotype modulation with possible increased synthesis of LDL-binding proteoglycans. To elucidate this, RNA was isolated from the 1-mm CCA segment proximal to either a constrictive collar (n=10) or nonconstrictive collar (n=8). Samples were pooled in pairs, and transcription levels were assessed on Affymetrix microarray chips.

To test for VSMC phenotypic modulation, we predefined gene sets with markers of the constrictive and the synthetic phenotypes. Also, to test for changes in the expression of proteoglycan core proteins, we predefined a gene set of proteoglycan core proteins, which have been associated with LDL retention.

Furthermore, we predefined a gene set of enzymes responsible for the synthesis of the glycosaminoglycan tetrascarachide linker, chondroitin sulfate-glycosaminoglycan and sulfation of chondroitin sulfate-glycosaminoglycans.

An over-representation analysis was performed using the GO-Elite software, and all 4 predefined gene sets were significantly over-represented in the analysis (Table). For validation of the over-representation analysis, we analyzed a selection of the genes by quantitative reverse transcriptase polymerase chain reaction (Figure 6). Biglycan (Bgn) was the only gene tested for which differential expression could not be validated by quantitative reverse transcriptase polymerase chain reaction (data not shown). Although Proteoglycan 4 (Prg4) has not been associated with lipoprotein retention, this proteoglycan core protein was the most affected in the microarray data set and was therefore included in the quantitative reverse transcriptase polymerase chain reaction analysis.

**LDL Retention in the CCA Depends on the Time Exposed to Disturbed Laminar Flow**

The gene expression data suggested that the induced lipoprotein retention was caused by structural changes in the arterial extracellular matrix. In that case, one would expect that lipoprotein retention would increase in magnitude with the time exposed to disturbed laminar flow. We therefore infused Atto 565–labeled LDL into mice implanted with constrictive collars...
either 5 hours or 2 weeks in advance (Figure 7A). Interestingly, artery segments exposed to disturbed laminar flow for 5 hours before LDL infusion retained only one third the amount of LDL compared with artery segments exposed to disturbed laminar flow for 2 weeks (Figure 7B). This result implies that although disturbed laminar flow induces some retention within the first day, this develops substantially over the course of weeks.

Retention of High-Density Lipoprotein at Natural and Artificially Induced Predilection Sites

Previous literature indicates that high-density lipoprotein (HDL) also interacts with arterial matrix.30–31 To test HDL retention in our systems, we repeated the experiments using Atto 680–labeled human HDL. At the 18-hour time point, circulating infused HDL was negligible (Figure 8A). As for LDL, HDL was retained both at the natural predilection sites of the aortic arch (Figure 8B) and proximal to the constrictive collar (Figure 8C and 8D). We conclude that lipoprotein retention induced by disturbed laminar flow is not specific for LDL.

Discussion

In the present study, we find that induction of disturbed laminar flow in the straight segment of the CCA transforms the nearby arterial wall into a lipoprotein-retaining site. This provides experimental evidence for the first step in a causal chain-linking flow patterns to atherosclerosis through regulation of lipoprotein retention. Importantly, the association between disturbed laminar flow and lipoprotein retention observed at natural predilection sites in several previous studies does not necessarily imply causation. Another possibility is that the arterial wall in inner curvatures and near branch points by way of their embryological development attains a different matrix composition when compared with other sites. To demonstrate the causal link between blood flow and lipoprotein retention, we induced disturbed laminar flow experimentally in the CCA using a method that has been previously central in determining the link between blood flow and atherosclerosis development.4 We show that disturbed laminar flow in normocholesterolemic mice induces binding of lipoproteins in the arterial media and intima, and that this is associated with relevant gene expression changes, indicating local phenotypic modulation of VSMCs, increased expression of mRNAs for lipoprotein-binding proteoglycan core proteins, and enzymes responsible for chondroitin sulfate–glycosaminoglycan synthesis and sulfation.

Our experiments were based on the fluorescent lipoprotein tracking technique that has been used previously to track lipoproteins into early atherosclerotic lesions in mice.32 By modifying this method with a near-infrared probe, whole tissue scans, and by applying it in normocholesterolemic mice, we showed that physiological retention in the thoracic aorta corresponds to the pattern of lesion development in mice models of atherosclerosis and humans. This finding is consistent with previous studies by Schwenke and Carew33 in rabbits using radioactive LDL-labeling techniques, and confirms that the ability of atherosclerosis-susceptible sites to bind high amounts of lipoproteins is a physiological characteristic that precedes the development of atherosclerosis. Furthermore, the result of this initial experiment served to validate the use of Atto 565- and Atto 680 NHS ester–labeled LDL in subsequent experiments because it was retained as anticipated from other model systems.

In humans, eccentric intimal thickening is consistently found at atherosclerosis predilection sites.34 Eccentric intimal thickening develops during early life35 and is thought to be a physiological adaptation of the artery wall to local flow dynamics, presumably involving the proliferation and extracellular matrix synthesis of VSMCs located in the basal

Figure 6. Validation of microarray results by quantitative reverse-transcriptase polymerase chain reaction. RNA isolated from the 1-mm segment proximal to either constrictive (n=5) or nonconstrictive collars (n=4) was used as starting material. Data are expressed as the binary logarithm of mRNA level fold change on constriction. *P<0.05 (Student t test).
intimal layer. The flow-mediated changes observed in the present study in mice support this idea although major structural changes, including intimal thickening, were not seen.

In early mouse atherosclerosis, fluorescently labeled LDL is mainly found among foam cells in the intima, but we found that the arterial media is a primary location of retained LDL in the normal mouse, both at natural predilection sites and after flow manipulation. Retained lipoproteins in the medial layer have also been reported earlier in nonlesioned arteries of rabbits using radioactive tracing techniques. Interestingly, the basal intimal layer is the principal site of lipid deposition before macrophage invasion in humans. One may speculate that in murine arteries, which lack an intimal layer, VSMCs in contact with retained LDL may thus function to attract the first inflammatory cells, and as the disease progresses, the intimal layer is expanded because of invasion to attract the first inflammatory cells, and as the disease progresses, the intimal layer is expanded because of invasion and matrix production. Once the intimal matrix is established, infused LDL will be trapped there before reaching the medial layer.

Lipoprotein retention at sites exposed to disturbed laminar flow is not specific for LDL, as we show that HDL is cleared from the circulation within 18 hours. The graph shows levels of human apoA1 in murine plasma in reinjected (control) mice (n=3) and 1 (n=4) and 18 (n=4) hours after HDL infusion as assayed with a human-specific apoA1 ELISA. Lines on graph indicate medians. Studies showing that not only LDL but also several types of macromolecules, including model antigens and immune complexes, localize preferentially to natural predilection sites for atherosclerosis. It is tempting to speculate that these specialized areas of the vasculature, induced by disturbed laminar flow, serve physiological roles in vascular homeostasis or host defense in the normal body. It also illustrates that the special role of LDL in inducing atherosclerosis is not explained by its preferential localization to the vascular wall, but by what occurs after LDL is retained.

The consequence of HDL retention is not yet clear. The most widely accepted role of HDL is in promoting cholesterol efflux from foam cells, but previous studies have indicated that HDL could also be atheroprotective by outcompeting its preferential localization to the vascular wall, but by what occurs after LDL is retained.
Further experiments are needed to clarify the upstream mechanisms relaying the mechanical force of blood into changes in lipoprotein binding of the arterial wall, as well as the relative importance of shear stress versus stretch in this process. Lowered wall shear stress has profound effects on endothelial cell phenotype, and it is conceivable that these may in turn influence local VSMCs to modulate and secrete lipoprotein-binding matrix through either direct contact or paracrine mediators. However, it is also possible that increased circumferential cyclic stretching of the artery wall, which is induced proximal to the constrictive collar and found at natural atherosclerosis-susceptible sites, directly affects phenotypic characteristics of VSMCs. The model of experimentally induced flow changes used in the present study may be a good model system to address these questions. Importantly, these mechanisms may be involved in not only creating the initial atherosclerosis-prone site but also subsequent growth of atherosclerotic lesions. Progression of atherosclerosis involves areas that were initially resistant to lipoprotein retention and atherosclerosis, and it is possible that local flow disturbances caused by plaque formation and leading to increased lipoprotein retainability in the neighboring regions could be among the driving mechanisms.

In conclusion, our data show that disturbed laminar blood flow augments the ability of the arterial wall to bind LDL in mice. This provides experimental evidence for the first step in the causal chain-linking disturbed laminar blood flow to initiation of atherosclerosis through increased lipoprotein retention as predicted by the response-to-retention theory.

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Disclosures

None

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Steffensen et al Disturbed Laminar Flow and Lipoprotein Retention

laminar flow and the ability of the nearby arterial wall to retain lipoproteins. Our experiments show that the introduction of disturbed blood flow in the straight segment of the common carotid artery increases the expression of mRNAs for proteoglycan core proteins and chondroitin sulfate side-chain assembly enzymes, modulates markers of vascular smooth muscle cell phenotype, and vastly augments the ability of the nearby arterial wall to retain lipoproteins. We believe that these findings document a key biomechanical mechanism that distinguishes "sulfate side-chain assembly enzymes, modulates markers of vascular smooth muscle cell phenotype, and vastly augments the ability of the nearby arterial wall to retain lipoproteins."
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Materials and Methods

Animal procedures
For LDL retention and microarray experiments, female B6 mice (C57BL/B6NTac, Taconic M&B, Ry, Denmark) were used, while female apolipoprotein E (apoE) deficient mice (B6.129P2-Apoemtm1Unc11, Taconic M&B, Ry, Denmark) were used to assess atherosclerosis. At the initiation of all experiments, mice were eight weeks old. Apoe deficient mice were fed a Western type diet for six weeks, while all other mice were fed rodent chow. All animal procedures were approved by the Danish Animal Experiments Inspectorate.

A constrictive or non-constrictive perivascular silastic collar (Helixmark, Helix Medical Inc., 1.5 mm in length, inner diameter of 0.31 and 0.50 mm, respectively) was implanted around the right common carotid artery (CCA) and secured with two 10-0 nylon ligatures (method adapted from Von der Thüsen et al.¹). For this procedure, mice were anesthetized with sevoflurane (induction 5%, maintenance 1.5-2.0%) and buprenorphine (1.5 mg/kg, subcutaneous). Buprenorphine was added to the drinking-water (6 mg/l) for three days following the surgical procedure. Mice were terminated by intraperitoneal injection of pentobarbital (250 mg/kg) and lidocain (20 mg/kg), perfused at 100 mmHg with Cardioplex for 30 seconds followed by 5 minutes perfusion with 4% phosphate-buffered formaldehyde through the left ventricle using the cut right atrium as route of drainage. Mice were then immersed in 4% phosphate-buffered formaldehyde for six hours at room temperature and stored in PBS at 4°C until further analysis.

LDL and HDL labeling and infusion
Human blood was collected in EDTA-coated tubes and centrifuged at 3,000 g for 10 min at 4°C. Plasma was isolated and mixed with KBr to a density of 1.21 g/l and layered in a KBr density gradient column (density layers: 1.006, 1.019, 1.063 and 1.21 g/ml). The column was centrifuged at 256,000 g for 18 hours, LDL was collected from the 1.063 g/ml density layer, purified on a PD10 column (GE Healthcare) with PBS and conjugated to either Atto 565 or Atto 680 N-Hydroxysuccinimide (NHS) ester fluorochrome (Sigma Aldrich) at pH 8.3, with 0.1 M NaHCO₃. Labeled LDL was purified on a PD10 column with PBS. Isolated Atto 565 NHS ester-labeled LDL was tested for purity on a TSKgel G3000SW column (Tosoh Bioscience). Five hours or two weeks after collar application, each mouse was infused with fluorescently labeled LDL (500 μg protein). Mice were perfusion fixated one hour or 18 hours after LDL infusion. The level of infused LDL was determined in plasma at one hour and 18 hours by a human apoB100 ELISA kit (27181, IBL International).

Human HDL (BT-914, Alfa Aesar) was conjugated to Atto 680 NHS ester fluorochrome (Sigma Aldrich) as described for LDL, and labeled HDL (500 μg protein) was infused into mice two weeks post collar application. Mice were then perfusion fixated one hour or 18 hours after HDL infusion. To determine the level of circulating HDL at these time-points, plasma apoA1 was measured by a human apoA1 ELISA kit (ab108804, abcam).

LDL immunoprecipitation
Protein G Sepharose beads (GE Healthcare) were cross-linked with either polyclonal rabbit anti-human apoB antibodies (Q0497, Dako) or the immunoglobulin fraction from non-immunized rabbits (X0903, Dako) (1 mg antibody per 30 µl beads) according to manufacturer’s instructions.

Atto 565 NHS ester-labeled LDL was incubated with either of the two types of beads (80 µl beads per mg LDL) at 4°C overnight at gentle rotation, and beads were removed by centrifugation at 5,000 g for 5 minutes. Proteins in LDL preparations post immunoprecipitation were separated by 3-8% Tris-acetate SDS-PAGE (Novex, Life Technologies) and 12% Tris-glycine SDS-PAGE, and Atto 565 NHS ester-labeled proteins were visualized using ImageQuant LAS 4000.

Tissue processing and image analysis
Thoracic aortas and CCAs were excised from mice fixated as described above. For Atto 680 quantification, periadventitial tissue was thoroughly removed. Thoracic aortas were opened longitudinally and mounted on microscope slides using Aquatex (Merck) and coverslips.
CCAs were treated the same way, but were unopened. Microscope slides were scanned using the 700 nm channel of a LiCor Odyssey Infrared Imaging System. Pixel values above background were summed for each CCA within a predefined 1 mm segment immediately proximal to the collar. Mice, which were not infused with LDL, served to determine background levels.

For Atto 565 quantification, arteries were cryoprotected in sucrose solutions (25% and 50% for 24 hours, respectively), embedded in Tissue-Tek O.C.T. Compound (Sakura) and snap frozen in liquid nitrogen. CCA cross-sections were collected at intervals of 100 µm starting from the proximal edge of the collar and ending at 500 µm proximal to the collar-edge. For aortic arches, longitudinal sections spanning the entire curvature were collected. Tissue sections were stained with DAPI (Sigma) and mounted with Slowfade Light Antifade (Invitrogen). Images of DAPI, green autofluorescence, and Atto 565 were acquired using an Olympus Cell-R wide-field fluorescence microscope system and the Xcellence Imaging Software (Olympus). To circumvent the problem of autofluorescence from the elastic laminae, all pixel values of the green (autofluorescence) channel were subtracted from the Atto 565 signal and the remaining signal was used for quantification.

For atherosclerosis quantification, CCA cryosections were prepared as for Atto 565 quantification and collected at intervals of 200 µm starting from the proximal edge of the collar and ending at 2 mm proximal to the collar-edge. Sections were stained using orcein and atherosclerosis was quantified by measuring the intimal area from the internal elastic lamina to the endothelium.

All quantitative image analysis was performed in ImageJ version 1.47t (NIH; http://rsb.info.nih.gov/ij/).

Microarray and quantitative reverse-transcriptase polymerase chain reaction
Anesthetized mice were flushed with saline via the left ventricle using the cut right atrium as route of drainage. The immediate 1 mm segment of the CCA proximal to the collar was excised and snap frozen in liquid nitrogen. RNA was isolated using RNeasy Micro Kit (Qiagen). RNA quality and quantity were measured using an Agilent Bioanalyzer. RNA samples were pooled in pairs and subsequent to cDNA synthesis, samples were loaded onto microarray chips (GeneChip MoGene 1.0 ST Array System, Affymetrix), preprocessed by the IterPLIER algorithm and analyzed using R. A Student’s t-test was made for each of the 28,850 genes on the chip. Gene sets of markers of vascular smooth muscle cell (VSMC) phenotype², proteoglycan (PG) core proteins associated with lipoprotein retention³ and enzymes responsible for CS-GAG synthesis and sulfation⁷ were pre-defined and an over-representation analysis (ORA) was performed using GO-Elite⁸ (http://www.genmapp.org/go_elite/) with default settings, all tested genes as denominator file, all genes with differential expression (p-value < 0.05 and transcription level difference of > 10%) as input file and the number of permutations set to 2000. Gene sets with z scores above 1.96 were considered significantly over-represented in the analysis.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed using the Brilliant III Ultra Fast SYBR Green QRT-PCR Master Mix (Agilent Technologies) on a Stratagene Mx3005P (Agilent Technologies) with the MxPro QPCR Software (Agilent Technologies). Cycling conditions were: 10 minutes at 50°C and 3 minutes at 95°C followed by 40 cycles of 20 seconds 95°C and 20 seconds at 60°C. Primer sets used were 5'-CTCCCCAGGAACATTGCC-3' and 5'-AACCGAAGGGACACTGAGCA-3' (Bgn), 5'-GATCAAGCCTGCGGTCG-3' and 5'-CCACCAGCAAACGAGAAGAT-3' (Lum), 5'-GAGGAGCCGTTTTTCTGACA-3' and 5'-AGGCGCTTCTCGTAAGTGAA-3' (Vcan), 5'-ACAGAAGCCGAGCATGAGAC-3' and 5'-GATGCAGTTTGGGTGATGCG-3' (Gapdh), 5'-AGTGGGATTTCCCTCTCC-3' and 5'-TGAATGGTGCCCACCTCTCTGA-3' (Prdg4, exon 7-9, NM_021400.3), 5'-ACCTGGGACATCAAAGGCTG-3' and 5'-TGCCCCATGGAGTCTCATT-3' (Csnalnact1), 5'-GAGGAGAGCAGGGCAACAT-3' and 5'-TGCAACTCTGCTCCGACCTTC-3' (Myh11), 5'-GAATCCACCCAGAACAAGGT-3' and 5'-GCCCTGATTGTCGCTTCTGA-3' (Myocd), 5'-CCAGGGATCAGGCTTGGCTT-3' and 5'-TGACTCATGGCTGCCCTTT-3' (Sppl), 5'-GCCCTGGCCTTGGTAGAAA-3' and 5'-ACTGTGGCCGTTAATTTTGC-3' (Gapdh). Gapdh was used as endogenous control and used for normalization.
Statistics
All statistical analysis was performed using the Prism statistical software (GraphPad, San Diego, CA). Two-sample nonparametric comparisons were performed using a Mann-Whitney test, and \( p < 0.05 \) was considered to be significant. For microarray and qRT-PCR experiments, data was assumed normally distributed, Student's \( t \)-tests were used, and \( p < 0.05 \) was considered to be significant. For comparison of multiple groups, Kruskal-Wallis tests followed by Dunn’s post test was used and \( p < 0.01 \) was considered to be significant. All images shown are examples of replicates and are representative for the respective groups.
References


