Functional Insights Into Novel Regulators of Plasma Lipids
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Hepatic Downregulation of Gpr146 in APOE*3-Leiden. CETP Transgenic Mice

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ABSTRACT

Introduction
GPR146 has recently emerged as a promising novel drug target for lipid-lowering therapies based on studies in mice. It is, however, unclear whether the protective effects observed in Gpr146 +/- mice also translate to humans. Here we use a model of acute hepatic Gpr146 downregulation in mice with humanized lipoprotein metabolism (APOE*3-Leiden.CETP) to investigate the effects on plasma lipid levels and atherosclerosis development.

Methods
Female APOE*3-Leiden.CETP mice on Western type diet were injected with AAV8-shRNA-Gpr146 or AAV8-shRNA-Control virus. Plasma lipids were measured every 4 weeks to monitor treatment effect on plasma lipids. 16 weeks after virus injection, the mice were sacrificed and atherosclerotic lesion size and severity was measured in the aortic valves.

Results
Compared to control, treatment with AAV8-shRNA-Gpr146 significantly decreased plasma cholesterol (~34%; P<0.0001) and triglyceride levels (~54%; P<0.0001) at 4 weeks after injection. This effect of the treatment on plasma lipids, however, became progressively smaller over time. Despite a significantly lower total cholesterol exposure (~33%; P<0.0001), no major differences in atherosclerosis lesion size or severity were observed between groups.

Conclusion
The biological and/or technical causes for the lack of protection against atherosclerosis in spite of the strong plasma lipid-lowering effect induced by hepatic Gpr146 downregulation cannot be answered with this study. This puzzling result may be due to potential side effects of the use of the specific shRNA sequence used, inflammatory effects of AAV8 as delivery system, or alternatively, due to biological effects of Gpr146 downregulation in not only the liver but possibly also other organs.
INTRODUCTION

Genome-wide association studies have shown that genetic variation in the GPR146 locus is associated with changes in high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol (1–3). Studies in mice have subsequently shown that complete loss of Gpr146 is associated with decreased plasma levels of both lipoproteins, which conferred protection against atherosclerosis development on a Ldlr−/− background (4). Although GPR146 inhibition has been proposed as a novel lipid-lowering drug target (4–7), it is currently not known whether these findings can be translated to humans. GPR146 is ubiquitously expressed, but studies in mice show that effects on plasma lipid metabolism are driven by the expression in the liver (4,7), suggesting that GPR146 is an interesting target for hepatic mRNA silencing strategies.

RNA interference is a rapidly developing therapeutic approach based on sequence-specific gene silencing mediated by small double-stranded RNAs (8,9). Its versatile nature opens the possibility of targeting disease related genes in the genome for which drugs are not yet available (10). Thus, RNA interference holds promise to greatly increase the available pharmacological tools in the battle against (cardiometabolic) diseases. The basis of RNAi-based therapy is to induce selective cleavage of the mRNA effectively leading to inhibition of target gene expression (11). This can be achieved, among others, with short interference RNA (siRNA) molecules carried in a vehicle or by using a viral vector to express the precursors of the siRNA known as short hairpin RNA (shRNA) (12). Very recently, siRNA therapeutic technology has reached the market for the field of dyslipidemia and cardiovascular disease with the European approval of an siRNA drug targeting PCSK9 to treat patients with heterozygous familial hypercholesterolemia (13). More siRNA-based drugs are undergoing preclinical studies and phase 1 or 2 clinical trials for lipid-lowering targets that e.g. include APOC3 (ClinicalTrials.gov: NCT03783377, (14), ANGPTL3 (ClinicalTrials.gov: NCT0372224) and LPA (ClinicalTrials.gov: NCT04270760).

Here we study the impact of acute hepatic Gpr146 downregulation using a shRNA approach in a humanized murine model of dyslipidemia: the APOE*3-Leiden.CETP transgenic mice. These mice express a dominant negative mutant of human APOE*3 which delays the clearance of very-low density lipoprotein (VLDL) while exhibiting intact LDL receptor (LDLR) expression and function. In addition, transgenic expression of human cholesteryl ester transfer protein (CETP) allows for transfer of cholesteryl esters in exchange of triglycerides from HDL to VLDL, which reduces HDL cholesterol and further contributes to humanized lipoprotein metabolism (5). The response of this mouse model to lipid-lowering treatments closely resembles the effects observed in humans (15,16). For the acute hepatic downregulation of Gpr146, we have used adeno-associated virus 8 (AAV8) to deliver a Gpr146-specific shRNA for mRNA degradation. The treatment with AAV8-shRNA-Gpr146 induced significant reductions of plasma lipid levels without conferring protection against atherosclerosis development.

MATERIALS AND METHODS

Animal experiments

All experiments were approved by the Institutional Animal Care and Use Committee from the University of Groningen (Groningen, the Netherlands). Animals were housed in individually ventilated cages with a light cycle of 12h and ad libitum access to food and water. Blood samples were collected after 4h of fasting in the morning by orbital bleeding under anesthesia with isoflurane.

Viral particles and virus dose titration

AAV8-GFP-U6-rm-Gpr146-shRNA (shRNA-Gpr146) and AAV8-GFP-U6-rm-scrambled-shRNA (shRNA-Control) were purchased from Vector Biolabs (Pennsylvania, USA, sequences available in Table S1). The shRNA sequence targeting Gpr146 mRNA in mouse and rat was validated by the manufacturer based on an shRNA screen showing 88% downregulation efficiency in vitro.

In a dose finding effort, male wildtype C57BL/6J mice (Charles rivers, the Netherlands) of 10 weeks of age (3 mice per dose), fed a chow laboratory diet (SNIFF, Bio Services, Uden, the Netherlands) were injected with 3 different doses of AAV8-shRNA-Gpr146: $1 \times 10^8$, $4 \times 10^8$ or $1 \times 10^9$ genome copies (gc) in a total volume of 100 µL of sterile PBS. The control group was injected with $4 \times 10^8$gc of the virus AAV8-shRNA-Control. Blood samples were collected at baseline (i.e., before injection) and 2 weeks after injection. Sacrifice was performed at 3 weeks after injection, when blood was obtained by cardiac puncture and organs were snap frozen in liquid nitrogen and stored at −80°C. A piece of liver was fixated with formalin for histological analysis.

Atherosclerosis induction with Western type diet

For the atherosclerosis study, 7-10 week-old female APOE*3-Leiden.CETP (17) mice were housed in pairs per cage. The animals used in the study were selected based on the anticipated increase in plasma lipids in response to a run-in period of 3 weeks on a Western type diet (WTD, 16% fat and 0.15% cholesterol, SNIFF, Bio Services, Uden, the Netherlands). After the run-in period, blood samples were collected to measure cholesterol and triglycerides in plasma. The animals with cholesterol levels
>9 mmol/L and triglycerides >2 mmol/L were used for treatment with AAV8–shRNA–Control or AAV8–shRNA–Gpr146. Responders were randomized to treatment based on plasma lipid levels and body weight to ensure similar fasting plasma total cholesterol, triglycerides and body weight in both groups at baseline (N=15 mice per treatment). The virus was injected intravenously via the orbital plexus at a concentration of 1x10^11 genome copies per mouse (based on results obtained from the titration experiment) in a total volume of 100 µL. Blood samples were collected at 4, 8 and 12 weeks after injection to monitor the effect of the treatment on plasma lipid levels. Sixteen weeks after virus injection, the mice were sacrificed under anesthesia with isoflurane. Plasma, liver, aorta, heart and adipose deposits were collected.

Plasma lipid measurements
Total cholesterol levels were measured with a colorimetric assay (11489232, Roche Molecular, Biochemicals) with cholesterol standard FS (DiaSys Diagnostic Systems) as reference following the instructions of the manufacturer. Triglyceride levels were measured using Trig/GB kit (Roche Molecular Biochemicals) with Roche Precimat Glycerol standard (Roche Molecular Biochemicals) as reference following the instructions of the manufacturer.

Non–HDL cholesterol and HDL cholesterol was determined by precipitation using 20% PEG-6000 (Sigma-Aldrich) in solution in glycine buffer (200 mM, pH 10). Cholesterol associated with HDL that remained in suspension after precipitation of apolipoprotein (apo)B-containing lipoproteins was measured as described above. Non–HDL–cholesterol was calculated by subtracting HDL–cholesterol from total cholesterol in plasma (18).

Fast Protein Liquid Chromatography (FPLC) analysis of lipoproteins
Cholesterol and triglyceride content of the main lipoprotein classes were determined using FPLC. The system encompasses a PU–980 ternary pump with an LG–980–02 linear degasser, FP–920 fluorescence and UV–975 UV/VIS detectors (Jasco, Tokyo, Japan). An extra PU–2080i Plus pump (Jasco, Tokyo, Japan) was used for in–line cholesterol PAP or triglyceride enzymatic reagent (Roche, Basel, Switzerland) addition at a flow rate of 0.1 mL/min. The plasma from individual mice was run over a Supersose 6 HR 10/30 column (GE Healthcare Hoevelaken, the Netherlands) using TBS pH 7.4, as eluent at a flow rate of 0.31 mL/min. Quantitative analysis of the chromatograms was carried out with ChromNav chromatographic software, version 1.0 (Jasco, Tokyo, Japan). The plots for individual FPLC profiles were generated with R version 3.6.1 (2019–07–05) and RStudio (19) using ggplot2_3.2.1, RColorBrewer_1.1–2, dplyr_0.8.3, and tidyr_0.8.3.

Liver histology and lipid measurements
Liver samples were fixed in Formalin and embedded in paraffin before staining with hematoxylin and eosin (H&E), using standard protocols (20). A qualitative histological analysis was performed to evaluate overall integrity of the tissue and different degrees of inflammation (absent, low and severe) and steatosis zonation 1–3 (absent, low, mild and severe).

Liver homogenates were prepared as 5% (w/v) in PBS to perform lipid extraction (21). In short, 100 µL of liver homogenate was extracted mixed with 700 µL of water and 3 mL of chloroform/methanol. After 30 min incubation 1.2 mL of water and 1 mL of chloroform was added, mixed and centrifuged for 10 min at 420g at room temperature. The chloroform layer was evaporated using nitrogen at 50°C, and lipids were resuspended in 1 mL of PBS with 2% triton X-100 and further analyzed for cholesterol and triglyceride content using the colorimetric kits as previously described (22).

Atherosclerotic lesion analysis
Atherosclerotic lesion analysis was performed according to the guidelines from the American Heart Association (23). At sacrifice, the hearts were fixated using 4% formalin solution in phosphate buffer (Klinipath BV, Netherlands), dehydrated with 70% ethanol and embedded in paraffin. The hearts were then cut in 4 µm cross–sections throughout the aortic root area, and hematoxylin–phloxin–saffron staining was performed for histological analysis. Atherosclerosis lesion analysis was performed in the aortic root of each heart, starting from the appearance of open aortic valve leaflets in four subsequent sections with 40 µm intervals. Lesion size was quantified, in a blinded fashion, by morphometric analysis of the valves using ImageJ software (version 1.53c). The severity score of each lesion was classified in 3 categories: no lesion (when no lesion found), mild (lesion types I–III) or severe (lesion types IV–V) according to the adapted guidelines for mouse from the American Heart Association, as described previously by Zadelaar et al., (2006).

RNA extraction and gene expression analysis by qPCR
50 mg of liver was homogenized in 1 mL of TRIpure reagent (Sigma–Aldrich) using zirconia beads and a bead beater with a program of 5,000 rpm for 30s twice and at 4°C. Total RNA extraction was performed by chloroform extraction followed by isopropanol precipitation. RNA pellets were washed twice with 70% ethanol before drying and resuspending in MiliQ RNAase free water. RNA concentration was measured using NanoDrop and adjusted to 1 µg/µL. cDNA synthesis of 1 µg of RNA was performed with M–MLV Reverse Transcriptase (Invitrogen) according to manufacturer’s instructions.

Effect of GPR146 hepatic downregulation
After virus injection, the mice were sacrificed under anesthesia with isoflurane. In short, 100 µL of liver homogenate was extracted mixed with 700 µL of water and 3 mL of chloroform/methanol. After 30 min incubation 1.2 mL of water and 1 mL of chloroform was added, mixed and centrifuged for 10 min at 420g at room temperature. The chloroform layer was evaporated using nitrogen at 50°C, and lipids were resuspended in 1 mL of PBS with 2% triton X-100 and further analyzed for cholesterol and triglyceride content using the colorimetric kits as previously described (22).

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qPCR analysis was done with FastStart SYBR Green Master (Roche) with primers specified in Supplementary Table S1, in the QuantStudio Real qPCR system (Applied Biosystems). Relative gene expression was calculated by the DeltaDeltaCT method using QuantStudio software (Version 1.2, Applied Biosystems) and normalized to a control sample and the expression levels of housekeeping genes (36b4 or Rplp0).

Analysis of hepatic enzymes
Mouse plasma samples were diluted 1:5 in Dulbecco’s Phosphate Buffered Saline (DPBS) and analyzed for aspartate transaminase (AST) and alanine transaminase (ALT) in the automatized instrument COBAS C (Roche) in the Laboratory of Biobinding Analysis from the University Medical Center Groningen.

Statistical analysis
Statistical analyses were performed with Graph Pad Prism (version 8). The differences of two groups were analyzed with unpaired two-tailed Student’s t-test. ANOVA test was used when more than one group was compared. All the analysis used alpha=0.05, and P values were considered significant when P<0.05 unless indicated otherwise.

RESULTS
AAV8-shRNA targeting Gpr146 decreases hepatic mRNA expression levels and decreases plasma cholesterol levels in wild-type C57BL/6J mice
Selection of the virus dose to downregulate Gpr146 in mouse liver was based on a titration study in which 3 dosages were tested in wild-type C57BL/6J male mice (outline of the experiment is detailed in Supplementary Figure S1A). Administration of 1x10¹¹ gc of AAV8-shRNA-Gpr146 did not change plasma cholesterol levels over the course of the 3-week experiment (Supplementary Figure S1B). However, at dosages of 4x10¹¹ gc and 1x10¹² gc, reductions of 22% (P=0.004) and 39% (P=0.004) were observed at 3 weeks after injection, respectively. The magnitude of this effect at the higher dosages resembles the 25-35% reduction of plasma total cholesterol levels observed in whole-body Gpr146⁻/⁻ mice compared to wild-type littermates (4,7). The FPLC cholesterol profile of the plasma samples collected 2 weeks after injection shows that at a dose of 4x10¹¹ gc, only a decrease in LDL cholesterol is observed while the 1x10¹² gc dose induced a decrease in both LDL and HDL cholesterol (Supplementary Figure S1C-D, respectively). No effect on plasma triglycerides was observed with any of the tested virus doses (Supplementary Figure S1E).

In line with the observed plasma lipid phenotypes, the 1x10¹¹ gc dose did not reduce Gpr146 mRNA expression in liver while 4x10¹¹ gc and 1x10¹² gc reduced it by 67% (P=0.004) and 69% (P=0.003), respectively (Supplementary Figure S2A). These data illustrate that the AAV8-shRNA-Gpr146 tool is effective at higher dosages. Since only the highest viral dose brought about decreases in LDL and HDL cholesterol, as seen in human studies (1,2) and similar to the phenotypes observed in whole-body as well as liver-specific knock-out Gpr146⁻/⁻ mice, we selected a dose of 1x10¹² gc per mouse for this study.

In light of the previously reported hepatotoxic effect of shRNA treatments (24), we measured plasma levels of hepatic enzymes ALT and AST in the mice treated with 1x10¹² gc of AAV8-shRNA-Gpr146 compared to controls in plasma collected at sacrifice. No differences between the groups were found (Supplementary Figure S2B-C). We also did not find significant differences in the gene expression levels of inflammatory markers Il1β, Il6, C668, Tnf and Il1a, in the livers of those mice (Supplementary Figure S2D). In agreement, the histological evaluation of these livers after H&E staining did not reveal presence of inflammation, steatosis or cellular abnormalities (Supplementary Figure 2SE). Based on all these initial evaluations of the AAV8-shRNA-Gpr146, we concluded that the selected AAV8-shRNA tool was effective to downregulate Gpr146 in mouse liver without triggering strong short-term side effects.
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Figure 1. Plasma lipid changes in response to AAV8-shRNA-Gpr146 treatment in comparison to control group treated with AAV8-shRNA-Control.

A. Plasma cholesterol values at different time points before and after treatment with AAV8-shRNA-Gpr146 virus to downregulate Gpr146 in liver in comparison with the group treated with AAV8-shRNA-Control. Mice were sacrificed (sac) after 16 weeks on the WTD diet.

B. Plasma triglyceride values at different time points before and after treatment with AAV8-shRNA-GPR146 virus to downregulate Gpr146 in liver in comparison with the group treated with AAV8-shRNA-Control. Mice were sacrificed (sac) after 16 weeks on the WTD diet.

C. FPLC cholesterol profile of individual plasma samples obtained 4 weeks after virus injection for mice treated with AAV8-shRNA-Gpr146 or AAV8-shRNA-Control.

D. FPLC triglyceride profile of individual plasma samples obtained 8 weeks after virus injection for mice treated with AAV8-shRNA-Gpr146 or AAV8-shRNA-Control.

E. Cumulative cholesterol exposure in mice treated with AAV8-shRNA-Gpr146 or AAV8-shRNA-Controls during the development of the experiment.

F. Gpr146 mRNA expression levels at the end of the atherosclerosis study, i.e., 16 weeks after virus injection.

Data represented as mean ± SEM, including individual data points (n=12-15/group). ns = non-significant.

Hepatic downregulation of Gpr146 protects against diet-induced dyslipidemia in APOE*3-Leiden.CETP mice

Compared to controls, a single injection of AAV8-shRNA-Gpr146 (1x10¹² gc) resulted in significant reductions in both total cholesterol and triglyceride levels in APOE*3-Leiden.CETP mice fed a WTD (Figure 1A-D), at 4, 8 and 12 weeks, following virus administration. FPLC analysis of individual plasma samples (Figure 1C-D) and HDL cholesterol quantification following PEG–6000 precipitation of plasma samples (Supplementary Figure S3A-B) revealed that the changes in plasma cholesterol and triglycerides were largely correlated with reductions in VLDL/LDL (non-HDL) cholesterol (Figure 1B-C and Supplementary Figure 3SA-B).

The plasma lipid reductions induced by the AAV8-shRNA-Gpr146 treatment, however, became progressively smaller over time, and at the moment of sacrifice, the differences between the groups were no longer statistically significant (Figure 1A-B). This may suggest an attenuation in the effectivity of the shRNA over time, as observed before in other studies using this technology (24). Accordingly, Gpr146 mRNA expression was not downregulated in the livers of AAV8-shRNA-Gpr146 treated mice compared to controls at the end of the experiment (Figure 1F).
Effect of GPR146 hepatic downregulation

Atherosclerosis lesion analysis in E3L-hCETP mice treated with AAV8-shRNA-Gpr146 and AAV8-shRNA-control.

A. Atherosclerosis lesion size in the aortic valves of mice treated with AAV8-shRNA-Gpr146 and AAV8-shRNA-Control.

B. Atherosclerotic lesion area across segments located at increasing distance from the aortic valves. This was determined by calculating the lesion area of four consecutive cross-sections starting from the appearance of open aortic valve leaflets.

C. Percentage of non-diseased segments in mice treated with AAV8-shRNA-Gpr146 and control group.

D. Atherosclerotic lesion categorization according to severity represented as percentage of segments with mild lesion (type I and II) and severe lesion (type III, IV, V) in mice treated with AAV8-shRNA-Gpr146 and control group.

E. Univariate linear regression analysis of the square root (SQRT) of atherosclerotic lesion size and the cumulative cholesterol exposure in mice treated with AAV8-shRNA-Gpr146 and control group.

Data represented mean ± SEM, including individual data points (n=12-15/group).

APOE*3-Leiden.CETP mice treated with shRNA–Gpr146 do not exhibit protection against atherosclerosis development

Despite a significantly lower total cholesterol exposure over the course of the experiment (Figure 1E), quantification of the atherosclerotic lesion area did not reveal reductions in lesion size and distribution across the aortic root in mice treated with shRNA–Gpr146 compared to controls (Figure 2A–B, Figure 3). The atherosclerotic severity score analysis revealed that treatment with shRNA–Gpr146 was associated with a significantly higher percentage of valves where no lesion was observed (t-test, P=0.03, Figure 2C); however, the overall assessment of mild and severe lesions did not show significant differences between groups (Figure 2D).

Multiple studies have shown that cholesterol exposure in the APOE*3-Leiden.CETP mouse closely correlates with atherosclerosis lesion size (25–27). Accordingly, linear regression analyses for total exposure to plasma cholesterol and the square root of the atherosclerotic plaque size show significant linearity in the control group but not in the group treated with the AAV8–shRNA–Gpr146 (Figure 2E). This suggests that other pro-atherogenic factors overruled the effect of reductions in plasma cholesterol in mice treated with AAV8–shRNA–Gpr146.

Figure 2. Representative examples of mild and severe atherosclerosis lesions in mice treated with AAV8–shRNA–Gpr146 and AAV8–shRNA-Control. Both groups presented high variation in lesion size and severity.

Figure 3. Atherosclerosis lesion analysis in E3L-hCETP mice treated with AAV8–shRNA–Gpr146 and AAV8–shRNA-Control.
APOE*3-Leiden.CETP mice treated with shRNA-Gpr146 do not present with clear metabolic or liver abnormalities

Compared to controls, shRNA-Gpr146-treated mice showed a small significant increased body weight gain in response to the WTD by the end of the experiment (Supplementary Figure S4A, ANOVA P<0.0001 followed by Sidak’s multiple comparisons test, at sacrifice P=0.02). This slight increase in body weight was not accompanied by a change in food intake (Supplementary Figure S4B) or plasma glucose levels (Supplementary Figure S3C). The liver weight and liver to body weight ratio was also similar in both experimental groups of mice, but two outliers with enlarged livers were observed only in the AAV8-shRNA-Gpr146 group (Supplementary Figure S4C-D). A qualitative histological analysis of the liver did not reveal significant differences in the incidence and degree of inflammation or steatosis between groups (Figure S5A-C). The levels of ALT were similar in both groups at 8 weeks after injection (Supplementary Figure S5F) while AST was significantly reduced in mice that received AAV8-shRNA-Gpr146 compared to controls (Supplementary Figure S5G, P=0.02). Hepatic lipid accumulation was not significantly different between groups (Supplementary Figure S5D-E).

DISCUSSION

In this study, we have used the APOE*3-Leiden.CETP mouse model to evaluate the effect of acute hepatocytic Gpr146 downregulation on plasma lipid levels and atherosclerosis. shRNA-mediated downregulation of hepatocyte Gpr146 resulted in very similar changes in plasma lipid levels as seen in whole-body knockout mice and hepatocyte-specific Gpr146−/− mice (4, 7). These findings underscore that GPR146 affects lipoprotein metabolism largely through its expression in the liver.

In our study, however, the differences observed in the plasma lipids between treatments became progressively smaller over time. This was in part due to an overall decrease in plasma lipid levels in the controls, an effect that has been reported previously in this mouse model on a high fat high cholesterol diet (28). In parallel, mice that had received AAV8-shRNA-Gpr146 presented with increasing plasma lipids over the time course of the experiment, suggests that the effectivity of the shRNA-mediated downregulation reduces over time, which was confirmed by the lack of Gpr146 mRNA downregulation at sacrifice.

Although it is reported that shRNA approaches can have sustained effects up to a year in the liver of mice (9), Grimm et al., (2006) found that a series of shRNA-expressing AAV8 targeting the human α-1 antitrypsin (hAAT) exhibited reduced effectiveness 30 days after a single injection in transgenic mice expressing hAAT. Furthermore, two sequences (out of the eight tested) induced only a temporary downregulation, after which increased expression of the target gene was observed compared to baseline levels (24), similar to what we observed in the livers of the AAV8-shRNA-Gpr146 treated mice. The authors indicate that a transient shRNA downregulatory effect seems to be due to the intrinsic regeneration capacity of the liver (29), which leads to the replacement of the targeted hepatocytes with new cells lacking shRNA (24, 30).

The lack of Gpr146 downregulation at the end of our study is compatible with this notion of previous hepatocyte regeneration and removal of the shRNA from the system. The stability of the downregulation and side effects of shRNAs also appear to depend on the dose and the specific shRNA sequence but not the targeted gene (24). Other studies indicate that the type of promoter driving the shRNA expression is also highly relevant. The AAV8 vectors with U6 promoter have been reported to rapidly lose the shRNA-mediated downregulatory effect due to transient liver injury (30). As an alternative, shRNA constructs with the H1 promoter have been suggested to perform better for long-term studies (30).

In their publication, Grimm and collaborators (2006) also describe the risk of serious hepatotoxic effects leading to mouse death related to shRNA treatment. Others have reported that hepatotoxicity can occur in the absence of a strong inflammatory reaction (31). In our study, we did not find evidence of marked liver pathology induced by the AAV8-shRNA-Gpr146 treatment at the level of histology, lipid accumulation or plasma levels of AST and ALT, in both wild-type C57BL/6J mice (sacrifice after 3 weeks) and APOE*3-Leiden.CETP mice (C57BL/6J background; sacrifice after 16 weeks). Hepatic enzymes were higher in AOE*3-Leiden.CETP mice compared to the levels reported for C57BL/6J of similar age for both AAV8-shRNA-Gpr146 and shRNA-Control groups, indicating that such an increase in the hepatic enzyme levels was not directly related to Gpr146 downregulation. In fact, we noted a small but significant reduction of AST levels in the APOE*3-Leiden.CETP mice treated with shRNA-Gpr146. Hepatic gene expression analysis in wild-type C57BL/6J mice collected 3 weeks after virus infection in our AAV8 titration experiment did not show significant increases in the gene expression of inflammatory genes compared to the control group, but we have no data on possible hepatic inflammatory effects in the atherosclerosis experiment. Of note, however, three mice from the AAV8-shRNA-Gpr146 group and none from the control group, died before the end of the atherosclerosis study for unknown reasons. Unfortunately, no necropsy was performed that may have revealed clues to a possible cause of death.
This brings the discussion to why we observed no effects on atherosclerosis despite a strong reduction in plasma lipid levels that was previously shown to reduce atherosclerosis significantly in this mouse model. Recent reports indicate that AAV8 vectors injected intravenously can have effects in multiple other organs besides liver, including heart and spleen (32). As GPR146 is reported to be expressed in different immune cells (33), one can speculate that Gpr146 downregulation there could have triggered systemic inflammatory effects that exacerbated atherosclerosis development in AAV8–shRNA–Gpr146 treated mice. Also, we cannot discard that local inflammatory effects on the aorta might have had an influence in the atherosclerosis outcomes found in our study. It is also possible that Gpr146 silencing could have induced detrimental effects in peripheral tissues, such as the adipose tissue, where GPR146 has been found to be involved in adipocyte differentiation (34). Technical reasons associated with shRNA-specific toxicity can also be considered based on current literature. Different studies have shown that shRNAs can have unspecific side effects associated with competitive inhibition of endogenous miRNA processing and functionality (24) or due to the activation of interferon systems (24,35,36). The design of our study, however, does not allow to investigate the mechanism underlying the absence of athero–protective effects of cholesterol lowering, which could be of technical nature related to shRNA toxicity (24,30), or also of biological nature related to unforeseen responses linked to downregulation of Gpr146.

In conclusion, our findings do not support for the use of the specific AAV8–shRNA–Gpr146 sequence deployed here at a dose of 1x10^12 gc as an appropriate strategy to prevent atherosclerosis in APOE*3Leiden.CETP mice. Understanding the technical and/ or biological causes for the lack of atherosclerosis protection in spite of the strong lipid–lowering effect, remains elusive and may help to steer future studies into the potential of hepaticotropic Gpr146 downregulation to reduce atherosclerosis. Possible alternatives to consider include the use of other promoters, siRNA/shRNA inhibition targeting different Gpr146 sequences or CRISPR/Cas9–mediated gene inactivation.

REFERENCES

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SUPPLEMENTARY DATA

A. Experimental design of the viral dose titration pilot study. Blood samples were collected prior and 2 weeks after virus administration. Mice were sacrificed 3 weeks after injection. All plasma samples were taken after 4h fast.

B. Plasma cholesterol at baseline and in response to the different viral doses of AAV8-shRNA-Gpr146. 2 and 3 weeks after virus injection.

C. Cholesterol FPLC of pooled plasma samples of mice (n=3) treated with AAV8-shRNA-Control-4x10^{11} gc or AAV8-shRNA-Gpr146-4x10^{11} gc. Samples were taken 2 weeks after virus injection.

D. Cholesterol FPLC of pooled plasma samples of mice (n=3) treated with AAV8-shRNA-Control-4x10^{11} gc or AAV8-shRNA-Gpr146-1x10^{12} gc. Samples were taken 2 weeks after virus injection.

E. Plasma triglycerides at baseline and in response to the different viral doses of AAV8-shRNA-Gpr146. 2 weeks after virus injection. Unlike plasma cholesterol, no significant differences in plasma triglycerides were found.

F. Data represent means ± SEM, including individual data points. Student t-test, *P<0.05, **P<0.01.

Supplementary Figure S1. AAV8-shRNA-Gpr146 induces hepatic downregulation of Gpr146 in a dose-dependent manner in C57BL/6J mice on a chow diet.
Chapter 6

Supplementary Figure S2. Plasma triglycerides and liver parameters measured in the dose determination pilot for AAV8-shRNA-Gpr146 in wild-type C57BL/6J male mice.
**Supplementary Figure S3.** Different plasma parameters measured at different time points during the development of the atherosclerosis study with AAV8-shRNA-GPR146 and AAV8-shRNA-Controls.

A. Non-HDL cholesterol determined after PEG-6000 precipitation in plasma samples obtained 4 and 8 weeks after virus injection
B. HDL cholesterol determined after PEG-6000 precipitation in plasma samples obtained 4 and 8 weeks after virus injection
C. Plasma glucose levels measured the day before sacrifice after 6 hours fast for mice treated with AAV8-shRNA-Gpr146 and AAV8-shRNA-Control.
D. Data represent means ± SEM, including individual data points (n=12-16/group).

**Supplementary Figure S4.** Mouse treated with AAV8–shRNA-Gpr146 show increased body weight gain in response to the Western Type diet without changes in food intake or liver weight.

A. Body weight change in response to Western type diet feeding in mice treated with AAV8–shRNA–Gpr146 and controls. Body weight differences across time were analyzed with ANOVA (Time P<0.0001 and Genotype P<0.0001) and followed by Sidak’s multiple comparisons test (only the body weight difference at sacrifice is significantly different P=0.02).
B. Average food intake per mouse during the atherosclerosis study for mouse treated with AAV8–shRNA–Gpr146 and AAV8–shRNA–Control.
C. Liver weight of mice treated with AAV8–shRNA–Gpr146 and AAV8–shRNA–Control. No statistical differences were found, but two remarkable enlarged livers are observed in only the AAV8–shRNA–Gpr146 group.
D. Liver weight to body weight ratio for the mice treated with AAV8–shRNA–Gpr146 and AAV8–shRNA–Control.
E. Data represent means ± SEM, including individual data points (n=12–15/group).
Supplementary Figure S5. No overall remarkable differences steatosis, inflammation, hepatic lipid content and liver enzymes in AAV8–shRNA–Gpr146 treated mice.