The gut microbiota in cardiovascular disease
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CHAPTER 4

A protective role for the antimicrobial peptide REG3γ in atherogenesis

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Abstract

Background
A role for the gut microbiota in the development of cardiovascular disease has recently been established. Therefore, understanding the mechanisms controlling the gut microbiota could be essential to identify factors in protection against atherogenesis. The production of mucus and excretion of antimicrobial peptides in the gut forms the first line of defense against infiltration of the gut microbiota into the intestinal epithelial barrier. Thus, antimicrobial peptides could play a protective role in atherogenesis by preventing infiltration of the gut microbiota into the intestinal epithelial barrier. Here, we investigated the contribution of the antimicrobial peptide REG3γ in the susceptibility to atherogenesis in mice.

Methods
We overexpressed a PCSK9 gain-of-function mutant in the liver of female Reg3γ−/− and WT littermate mice via an AAV-delivery system and fed these mice a high-fat, high-cholesterol diet (HFC) for 11 weeks. During time of sacrifice heart, aortic arch, blood, intestine and fecal samples were collected to determine atherosclerosis development.

Results
Hepatic expression of PCSK9 gain-of-function mutant resulted in a strong reduction in the LDL receptor (LDLR) in the liver, which coincided with hypercholesterolemia in both Reg3γ−/− and WT mice. Analysis of atherosclerotic lesion size in the aortic root demonstrated a 28% increase in lesion size in Reg3γ−/− mice with respect to WT littermates. These effects are independent of alterations in blood lipid levels, intestinal permeability or alterations in microbiome composition.

Conclusions
The antimicrobial peptide REG3γ plays a protective role against the development of atherosclerosis.
**Introduction**

Atherosclerosis is one of the leading causes of death worldwide with a multifactorial etiology that includes a role for genetics, dietary intake and inflammation (Krauss et al., 2000; Libby et al., 2011; Lusis et al., 2004). Recently, the gut microbiota has been identified as an additional player in atherogenesis (Koeth et al., 2013; Li et al., 2016; Tang and Hazen, 2014; Chapter 3). Both a disturbance in microbiota composition (Koren et al., 2011; Ott et al., 2006) and in the production of microbiota-derived metabolites, such as trimethylamine-oxide (TMAO) and short-chain fatty acids (SCFA’s) (Aguilar et al., 2014; Koeth et al., 2013) has been implicated in atherogenesis.

Studies have shown that increased infiltration of luminal bacteria into the intestinal epithelial barrier results in the translocation of bacteria or endotoxins across the intestinal epithelial lining into the systemic circulation (Wang et al., 2016). This increase in endotoxins and bacteria into the systemic circulation is linked to the atherosclerotic disease process (Koren et al., 2011; Li et al., 2016). In addition, taxonomies of the gut microbiota have been found in atherosclerotic plaques of patients with symptomatic atherosclerosis (Koren et al., 2011; Ott et al., 2006) and injection of LPS has been shown to exacerbate atherogenesis in ApoE\(^{-/-}\) mice (Yin et al., 2013). Taken together, these findings indicate that controlling the infiltration of luminal bacteria into the intestinal epithelial barrier could be of importance in protecting against atherosclerosis.

The epithelial lining of the intestine provides the first line of defense against the infiltration of bacteria from the gut lumen into the intestinal epithelial barrier (Brandsma et al., 2015; Loonen et al., 2013; Vaishnava et al., 2011; Wang et al., 2016). This physical-chemical barrier consists of a mucus layer in which Paneth cells excrete antimicrobial peptides, such as the C-type lectin REG3\(\gamma\). REG3\(\gamma\) prevents the infiltration of gram-positive bacteria into the intestinal epithelial barrier and Reg3\(\gamma\)^{-/-} mice display enhanced infiltration of gram-positive bacteria into the intestinal epithelial barrier (Loonen et al., 2013; Vaishnava et al., 2011; Wang et al., 2016). Several studies have reported the importance of these antimicrobial peptides, including REG3\(\gamma\) in the protection against leakage of endotoxins or bacteria from the gut lumen into the systemic circulation in the development of the metabolic syndrome (Su et al., 2016; Veilleux et al., 2015; Wang et al., 2016). However, it has not been studied whether
increased infiltration of the gut microbiota into the intestinal epithelial barrier plays a role in atherogenesis. Here, we used Reg3γ−/− mice to investigate the importance of bacterial infiltration into the intestinal epithelial barrier to the atherosclerotic disease process. We show that atherogenesis is accelerated in Reg3γ−/− mice, indicating that preventing bacterial infiltration is an important factor in controlling atherogenesis.

Material and Methods

AAV-production
HEK293T cells were transfected with helper plasmid, adenoviral plasmid and pAAV/D377Y-mPCSK9 plasmid in a 2:1:1 ratio using polyethylenimine transfection. Cells were harvested after 60 hours of culturing at 37°C, 5% CO2. The recombinant PCSK9-gain-of-function-AAV (PCSK9-GOF-AAV) was isolated from the cells by two freeze-thaw cycles. PCSK9-GOF-AAV was purified from the lysate by iodixanol centrifugation, desalted, concentrated by spin filter and stored at -80°C.

Animal experiment
All animal studies were performed with approval by the University of Groningen Ethical Committee for Animals Experiments, which adheres to the principles and guidelines established by the European Convention for the Protection of Laboratory Animals. Experiments were carried out on female Reg3γ−/− mice (B6.129-Reg3gtm1.1Lvh/J; Jackson Laboratory, Bar Harbor, USA, ME) and WT littermates, bred inhouse. Mice were housed in groups in Individual Ventilated Cages and maintained on a 12-hour light/12-hour dark cycle with ad libitum access to food and water. Reg3γ−/− mice and WT littermates were injected via the orbital vein with 2.0x10^10 vector genomes of PCSK9-GOF-AAV at 12 weeks of age (Bjorklund et al., 2014). The diet was switched from standard chow to high-fat cholesterol diet (HFC; 60% kcal fat, 0.25% cholesterol, Research Diets) 2 days after AAV-injection. Reg3γ−/−(PCSK9) and WT(PCSK9) mice were fed a HFC diet for 11 weeks. Blood was collected after 2 and 7 weeks to measure plasma lipid levels. Fecal samples were collected on the day prior to sacrifice. Mice were sacrificed using cardiac puncture, blood was collected in EDTA-coated tubes, spun down at 1000g for 10 min at 4°C and plasma was stored for further analysis. Aortic arches were removed and frozen in liquid
nitrogen. Hearts were embedded in OCT, frozen on dry ice in isopentane and stored at -80°C until further analysis. Intestines were removed and duodenum, ileum, and colon were dissected and snap-frozen in liquid nitrogen or fixated in carnoy fixative (Johansson and Hansson, 2012).

**Fecal transplantation experiment**
The microbiota of Casp1<sup>-/-</sup> mice (a gift from Prof. Netea (Joosten et al., 2009) (B6N.129S2-Casp1tm1Flv/J)) was transplanted into Reg3γ<sup>-/-</sup> and WT mice (Fig 4B). Fecal transplantation was performed as previously described (Chapter 3). In short, at the age of 10 weeks mice were treated with antibiotics for 10 days, followed by transfer of bedding from Casp1<sup>-/-</sup> mice into the cages of Reg3γ<sup>-/-</sup> or WT mice for 7 days. Mice were then injected with 2.0x10<sup>10</sup> vector genomes of PCSK9-GOF-AAV (Bjorklund et al., 2014). The diet was switched from a standard chow diet to a HFC diet 2 days after AAV-injection and Reg3γ<sup>-/-</sup>(PCSK9) and WT(PCSK9) mice remained on this diet for 13 weeks. Blood was collected at week 2 and week 7 after the start of the HFC intervention to determine plasma lipid levels and mice were sacrificed at week 13 of HFC feeding and organs were collected as described above.

**Microbiota composition**
Microbiota composition was determined as described in Chapter 3. In short, fecal samples were collected 1 day prior to sacrifice and immediately snap-frozen, fecal DNA was isolated and sequenced using Illumina MiSeq paired-end reads. Amplicons were generated by 16S sequencing targeting the hypervariable V4 region. An OTU-table was made by closed-reference OTU-picking at 97% making use of Greengenes13_8 reference database. Weighted Unifrac distances were determined in Qiime and plotted into PCoa plots in R. Determination of statistically significantly different taxonomies was classified by MaAsLin analysis (Morgan et al., 2012).

**Analysis of plasma parameters**
Plasma triglycerides and total cholesterol were determined by commercially available kits (Roche/Hitachi, Basel, Switzerland).
**Gut permeability Assay**

Gut permeability was measured in fed mice at time of sacrifice. Mice received 0.6 mg/g bodyweight of FITC-conjugated dextran (Sigma-Aldrich, St Louis, USA) by oral gavage and blood was collected via cardiac puncture after 4 hr. The concentration of FITC was determined in plasma by fluorometry at 488 nm as described in Chapter 3.

**Histological analysis of atherosclerosis**

Hearts were cut into sections of 7 µm at the aortic root, after which serial cross-sections of every 42 µm were stained with toluidin blue. Slides were scanned with a Hamamatsu slide scanner and plaque size was measured in a blinded fashion using image scope software (Leica Aperio Imagescope, Wetzlar, Germany) and was presented as the sum of 3 valves.

**Quantitative real-time PCR**

Total RNA from aortic arches was isolated using Qiazol reagent and cDNA was synthesized using the Transcriptor Universal cDNA Master kit (Roche, Mannheim, Germany). Real-time PCR was performed with a 7900HT PCR system (Applied Biosystems, Foster city, CA, US) using SYBR Green Master Mix reagent (Roche, Mannheim, Germany). Each sample was run in triplicate and normalized to PPIA as housekeeping gene. We calculated fold changes in gene expression normalized to PPIA by the ΔΔCT method using the equation 2-ΔΔCT. The results are shown as fold changes with respect to the compared to the control group. Primer sequences are listed in Supplemental Table 1.

**Statistical analysis**

All data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 5 Software (Graphpad Software, San Diega, CA, USA). All data were tested for normality by d’Agastino and Pearson omnibus normality test. For normally distributed data Student’s T-test was used. For non-normally distributed data, non-parametric Mann-Whitney U test was used. Data were considered significant if p < 0.05.
Results

PCSK9-GOF-AAV injection promotes hyperlipidemia
To understand whether infiltration of bacteria into the intestinal epithelial barrier is involved in atherogenesis, we first injected Reg3γ−/− and WT littermate mice with 2.0x10^10 particles of adeno-associated virus (AAV) containing a PCSK9 gain-of-function mutant (AAV-PCSK9-GOF). Reg3γ−/−(PCSK9) and WT(PCSK9) mice were then fed a HFC-diet for 11 weeks to induce atherogenesis (Bjorklund et al., 2014; Ishibashi et al., 1993). Injection with AAV-PCSK9-GOF resulted in massive reduction of LDLR in the livers of Reg3γ−/− and WT mice (Figure 1A, B), reaching levels which are comparable to LDLR levels in Ldlr knockout (Ldlr−/−) mice (Figure 1A, B). As a result, hepatic expression of PCSK9-GOF raised plasma cholesterol levels over time (Figure 1C) and to levels equivalent to Ldlr−/− mice fed a HFC-diet (Figure 1D). Thus, AAV-PCSK9-GOF injection together with HFC feeding created a hyperlipidemic mouse model comparable to the atherosclerosis-prone Ldlr−/− mice. We observed no differences in plasma total cholesterol levels (Figure 1C) and in the distribution of cholesterol among lipoprotein particles (Figure 1E) between Reg3γ−/−(PCSK9) and WT(PCSK9) mice. Furthermore, ablation of Reg3γ did not affect body weight (Figure 1F) and plasma triglyceride levels (Figure 1G), indicating that metabolic parameters are similar between the two groups.

Increased bacterial infiltration promotes atherosclerosis
Next, we assessed atherosclerosis development in both groups by analyzing the atherosclerotic lesion size in the aortic root of HFC-diet fed Reg3γ−/−(PCSK9) and WT(PCSK9) mice. Atherosclerotic lesion size was increased by 28% in Reg3γ−/−(PCSK9) mice compared to WT(PCSK9) mice (Figure 2A, B; p<0.05). This was not accompanied by increased expression of the inflammatory genes Tnfα, Mcp-1, Icam-1, iNos and Cd68 in the aortic arch of Reg3γ−/−(PCSK9) with respect to WT(PCSK9) mice (Figure 2C-G). Thus, Reg3γ−/−(PCSK9) mice show an increase in atherosclerosis development in the aortic root, without affecting inflammatory gene expression in the aortic arch.
**Figure 1 - Blood lipid levels are not altered in Reg3γ/-/- mice.**

Female Reg3γ/-/- mice or WT littermates were injected with PCSK9-GOF-AAV and fed a HFC diet for 11 weeks. (A) Gene expression analysis of Reg3γ. (B) Western blot analysis of LDLR in liver. (C) Quantification of western blot analysis. (D) Plasma total cholesterol concentrations over time. (E) Total cholesterol in plasma at t=11 weeks. (F) Body weight, (G) Triglycerides in plasma at t=11 weeks (H) FPLC profile at t=11 weeks. Throughout, data represent mean ± S.E.M. *P<0.05; by unpaired two-tailed Student’s t-test.
**Increased atherogenesis in Reg3γ−/− mice is independent of intestinal permeability**

To understand whether the enhanced atherogenesis in Reg3γ−/−(PCSK9) mice is caused by aberrant intestinal health, we analyzed intestinal inflammation and permeability. Histological analysis of the ileum showed no inflammatory phenotype in Reg3γ−/−(PCSK9) compared to WT(PCSK9) mice (Figure 3A, B). This was further confirmed by gene expression analysis of inflammatory markers in the ileum, which showed that Reg3γ deficiency does not affect the expression of the pro-inflammatory genes Mcp1, Ifn-γ, Il1β, Tnf-α and Ccl5 (Figure 3C-H). In addition, intestinal permeability, as measured by the FITC-dextran (FD4) in vivo permeability assay, was not affected by depletion of Reg3γ (Figure 3F). These results indicate that intestinal barrier function is not compromised in Reg3γ−/−(PCSK9) mice and thus cannot explain the increase in atherogenesis seen in these mice.

**Reg3γ−/− mice do not display microbiota dysbiosis**

To exclude a role for a disturbed gut microbiota composition as driving factor for the increased susceptibility to atherogenesis in Reg3γ−/−(PCSK9) mice, we analyzed fecal microbiota composition of HFC-diet fed Reg3γ−/−(PCSK9) and WT(PCSK9) mice by 16S rDNA sequencing. Weighted UniFrac distances of 16S rDNA sequences demonstrated clustering between the Reg3γ−/−(PCSK9) mice, whereas the microbiome of WT(PCSK9) mice was more diverse (Figure 4A). To understand whether there are specific taxonomies that are altered between Reg3γ−/−(PCSK9) mice with respect to WT(PCSK9) mice, we investigated the microbiome on the genus level, and found no major differences in the top-20 of most abundant taxonomies (Figure 4B). In addition, Maaslin analysis failed to detect statistical significant differences between the taxonomies of the gut microbiome and the genotype of the mice indicating that the microbiota composition is not markedly altered by the loss of REG3γ (Figure 4C).

**AAV-PCSK9-GOF injection does not result in hepatic LDLR knockdown and a hyperlipidemic mouse model**

As our data emphasizes a protective role for REG3γ in atherogenesis, we next assessed whether a microbiome harboring bacterial species prone
Figure 2 - Increased bacterial infiltration promotes atherosclerosis

Female Reg3γ−/− mice or WT littermates were injected with PCSK9-GOF-AAV and fed a HFC diet for 11 weeks. (A) Representative images of the aortic root stained by toluidin blue for determination of the lesion area. (B) Quantification of atherosclerotic root lesion area. (C-G) Gene expression in the aortic arch. (C) Tnfα. (D) Mcp-1. (E) Icam-1. (F) Inos. (G) Cd68. Throughout, data represent mean ± S.E.M. *P<0.05; by unpaired one-tailed Student’s t-test.
to infiltrate the intestinal epithelial barrier (e.g. mucispirillum schaedleri, Attaching Invading Eschericia Coli) (Loy et al., 2017; Martinez-Medina et al., 2014) is able to further advance atherogenesis in Reg3γ−/− (PCSK9) mice. We have recently shown that Ldlr−/− mice transplanted with the pro-inflammatory microbiome of Caspase 1−/− (Casp1−/−) mice display an increased abundance of the taxonomies Bilophila, Streptococcus and Mucispirillum (Chapter 3). As these mice show advanced atherogenesis (Chapter 3), we hypothesized that atherogenesis would be more severe

**Figure 3 – Intestinal inflammation and permeability is not affected in Reg3γ−/− mice.**

Female Reg3γ−/− mice or WT littermates were injected with PCSK9-GOF-AAV and fed a HFC diet for 11 weeks. (A) H&E staining of ileum. (B-F) Gene expression analysis of inflammatory markers in the ileum. (B) Mcp-1. (C) Ifn-γ. (D) Il-1β. (E) Tnf-α. (F) Ccl5. (G) FITC dextran in vivo permeability assay. Throughout, data represent mean ± S.E.M. *P<0.05; by unpaired one-tailed Student’s t-test.
Figure 4 – Reg3γ/- mice do not show disturbances in microbiota composition.

Female Reg3γ/- mice or WT littermates were injected with PCSK9-GOF-AAV and fed a HFC diet for 11 weeks. (A) Principal-coordinate analysis plot of Weighted
in Casp1−/− microbiome-transplanted Reg3γ−/−(PCSK9) mice compared to Casp1−/− microbiome-transplanted WT(PCSK9) mice. Thus, we performed a fecal microbiota transplantation in Reg3γ and WT littermate mice (Figure 5A) and transduced the mice with a PCKS9-GOF-AAV to provoke atherogenesis under HFC-feeding conditions. A new batch of AAV-PCSK9-GOF was generated; however, this virus batch did not result in depletion of hepatic LDLR (Figure 5B, C) and hypercholesterolemia (Figure 5D-F) in Casp1−/− microbiome-transplanted Reg3γ−/−(PSCK9) and WT (PCSK9) mice. As a consequence, atherosclerosis development could not be studied in Casp1−/− microbiome-transplanted Reg3γ−/−(PSCK9) and WT (PCSK9) mice, since it is very unlikely that atherosclerotic plaques have developed under these conditions (Ishibashi et al., 1993).

Discussion
To explore whether infiltration of luminal bacteria into the intestinal epithelial barrier plays an important role in the atherosclerotic disease process, we investigated atherogenesis in Reg3γ−/− and WT littermate mice expressing a PCSK9-GOF mutant and fed a HFC-diet for 11 weeks to provoke atherogenesis. Our data show that atherogenesis is increased by 28% in Reg3γ−/−(PCSK9) compared to WT(PCSK9) mice (Figure 2A-B; p<0.05). This was not associated with a difference in plasma lipid levels (Figure 1E,G), impaired intestinal permeability (Figure 3G) and gut microbiota dysbiosis (Figure 4C). Our findings unravel an atheroprotective role for REG3γ and together with previous reports identifying a protective role for antimicrobial peptides in the etiology of the metabolic syndrome (Su et al., 2016; Veilleux et al., 2015; Wang et al., 2016) emphasize the importance of antimicrobial peptides in the prevention of cardio-metabolic diseases.

It has previously been shown that infiltration of bacteria into the intestinal epithelial barrier can contribute to increased intestinal inflammation

*UniFrac distance on the basis of 16S-rDNA-encoding sequences in feces collected from Reg3γ−/− mice and WT littermates fed a HFC diet for 11 weeks. (B) Stacked bar graph representing the 20 most abundant taxonomies. (C) Top 20 most different taxonomies from MAaslin analysis. Throughout, data represent mean ± S.E.M. *P<0.05; by unpaired one-tailed Student’s t-test.
Figure 5 - PCSK9-GOF-AAV injection did not promote hypercholesterolemia following fecal transplantation.
and increased leakage of endotoxins or bacteria into the systemic circulation (Loonen et al., 2013; Martinez-Medina et al., 2014; Wang et al., 2016). However, increased atherogenesis in Reg3γ−/−(PCSK9) mice was not related to a disruption in intestinal integrity, as we did not observe differences in intestinal inflammation and permeability between Reg3γ−/− (PCSK9) and WT(PCSK9) mice (Figure 3). However, the lack of differences in the intestinal permeability in Reg3γ−/− mice as measured by the FITC-dextran assay does not exclude translocation of bacteria or endotoxins from the gut lumen into the systemic circulation. Indeed, previous studies have shown that translocation of bacteria from the gut lumen into the mesenteric lymph nodes and liver can occur without significant differences in paracellular transport of bacteria (Wang et al., 2016). In line with this, it has been shown that bacterial transport may occur via the uptake of bacteria by CX3CR1hi expressing mononuclear phagocytes from the lamina propria. These cells can consequently migrate to the mesenteric lymph nodes (Diehl et al., 2013), where the bacteria can reach the systemic circulation via the lymphatic ducts. However, future experiments measuring the presence of bacteria in the circulation and the transport of bacteria via CX3CR1hi mononuclear phagocytes in Reg3γ−/− mice are needed to understand whether translocation of bacteria from the gut lumen to systemic circulation contributes to increased atherosclerosis in Reg3γ−/− mice. Next to bacteria, endotoxins from the gut lumen can also transport via transcellular routes to the systemic

Female Ldlr−/− mice aged 12 weeks were exposed to fecal microbiome derived from Casp1−/− or Ldlr−/− mice for 13 weeks while fed a chow diet or a HFC diet. (A) Experimental setup of fecal microbiota transplantation. Female Reg3γ−/− or WT littermates were orally gavaged with a cocktail of broad spectrum antibiotics for a period of 10 days to suppress intestinal microbes. This was followed by daily transfer of used bedding material from cages housing Casp1−/− mice to cages housing Reg3γ−/− or WT littermates for 1 week. Reg3γ−/− or WT littermates were then injected with 2.0x1010 PCSK9-GOF-AAV and cohoused with Casp1−/− mice in a 3:2 ratio. 2 days post AAV-injection Reg3γ−/−(Casp1−/−) mice and WT(Casp1−/−) mice were switched to a HFC diet for a period of 13 weeks. (B) Western blot analysis of LDLR in liver. (C) Quantification of western blot analysis. (D) Plasma total cholesterol concentrations over time. (E) Total cholesterol at t=13 weeks (F) Triglycerides at t=13 weeks. (G) Body weight. Throughout, data represent mean ± S.E.M. *P<0.05; by unpaired two-tailed Student’s t-test.
circulation. Endotoxins and in particular lipopolysaccharide can be taken up by intestinal epithelial cells where they can bind to chylomicrons, which are abundantly formed during high fat diet feeding (Ghoshal et al., 2009). Consequently the chylomicrons can transport together with the endotoxins to the basolateral side, thereby facilitating the transport of endotoxins from the gut lumen into the systemic circulation (Ghoshal et al., 2009). Endotoxemia was however not involved in the increased susceptibility of Reg3γ−/− mice for systemic inflammation in alcoholic steatohepatitis (Wang et al., 2016). Future experiments are needed to understand if endotoxemia contributes to increased atherogenesis in Reg3γ−/−(PCSK9) mice.

We did not detect a disturbance in microbiota composition between Reg3γ−/−(PCSK9) and WT(PCSK9) mice (Figure 4). This is in accordance with previous literature showing that Reg3γ−/− mice do not harbor a dysbiotic gut microbiota compared to WT mice (Loonen et al., 2013; Vaishnava et al., 2011). To elucidate the protective contribution of the antimicrobial peptide REG3γ against dysbiosis-induced atherosclerosis, we introduced the pro-inflammatory gut microbiota of Casp1−/− mice into antibiotic-treated Reg3γ−/− and WT mice via a cohousing approach (Figure 5A) (Elinav et al., 2011; Henao-Mejia et al., 2012, Chapter 3). Reg3γ−/− (Casp1−/−) and WT (Casp1−/−) mice were then injected with a PCSK9-GOF-AAV (Bjorklund et al., 2014) to accelerate atherogenesis. However, total cholesterol levels after 13 weeks of HFC-diet feeding were only mildly increased to 9 mmol/L in contrast to 35 mmol/L in Reg3γ−/−(PCSK9) or WT(PCSK9) mice (Figure 5D, E). In addition, western blot analysis of LDLR in liver homogenates of Reg3γ−/−(Casp1−/−) and WT(Casp1−/−) mice did not show a downregulation of LDLR (Figure 5B,C). For injection of WT(Casp1−/−) or Reg3γ−/−(Casp1−/−) a new batch of PCSK9-GOF-AAV was generated, a lack of downregulation in LDLR in the liver of these mice indicates that, this batch of PCSK9-GOF-AAV did not significantly promote lysosomal degradation of hepatic LDLR. A limited response to the injection of the PCSK9-GOF-AAV can possibly be explained by a reduction in the proportion of infectious particles generated in the second batch of AAV production.

Overall our study shows that the antimicrobial peptide REG3γ plays a protective role in the development of atherosclerosis. Future experiments need to be conducted to understand how REG3γ protects atherosclerosis development. Especially, investigation of transcellular routes for
transportation of endotoxins and bacteria should be considered. In addition, it is important to repeat the fecal transplantation experiment to understand the protective role of antimicrobial peptides during conditions in which the gut microbiome is disturbed and can further promote atherosclerosis development. A better understanding of the function of antimicrobial peptides and the protection they confer during dysbiosis in the etiology of atherosclerosis may possibly lead to novel targets in protection against atherosclerosis, either by improving antimicrobial peptide production or by specifically targeting specific microbial species invading the intestinal epithelial barrier.
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