Chapter 6

Hepatic overexpression of chemerin does not affect nonalcoholic steatohepatitis and insulin resistance in low-density lipoprotein receptor knock-out mice

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In preparation
Abstract

Pro-inflammatory adipokines are believed to play a role in the progression of nonalcoholic fatty liver disease (NAFLD). Chemerin is a novel adipokine involved in the modulation of inflammation and is strongly correlated with nonalcoholic steatohepatitis (NASH), a form of NAFLD characterized by steatosis, hepatocyte injury, inflammation and fibrosis. However, it is still unknown if chemerin is causally involved in its etiology. To investigate this, we first examined whether chemerin signaling affects inflammation in macrophages and hepatocytes. Ablation of the chemerin receptor, chemokine-like receptor 1, increased \textit{Il-6}, \textit{Il-1β} and \textit{Tnfα} gene expression in macrophages, whereas stimulation of primary hepatocytes with recombinant chemerin reduced the expression of \textit{Il-1β} and \textit{Tnfα}. These results suggest that chemerin may protect against inflammation in NASH. To investigate if chemerin alleviates NASH \textit{in vivo}, we overexpressed chemerin in a mouse model for NASH, the low-density lipoprotein receptor knock-out (\textit{Ldlr}⁻⁻) mice, using a self-complementary adeno-associated virus. Overexpression of chemerin did not affect hepatic steatosis on a chow diet or a HFC diet. As expected, feeding \textit{Ldlr}⁻⁻ mice a HFC diet increased the expression of pro-inflammatory (\textit{Il-1β}, \textit{Tnfα} and \textit{Cd68}) and pro-fibrotic (\textit{αSma} and \textit{Col1a1}) genes, but this was not attenuated by chemerin overexpression. Since chemerin has also been implicated in the development of insulin resistance, we assessed the level of glucose and insulin tolerance by performing a glucose and insulin tolerance test. However, no differences in insulin resistance were observed between the virus-groups. Taken together, these results suggest that chemerin is not involved in the etiology of NASH and does not contribute to insulin resistance.
Introduction

Nonalcoholic steatohepatitis (NASH) is part of a spectrum of nonalcoholic fatty liver diseases (NAFLDs). It is characterized by the presence of steatosis, hepatocyte injury, inflammation and fibrosis [1]. With no cures currently available, NASH is one of the worst problems facing the obese population. When NASH becomes too severe this can eventually lead to scar tissue formation and perturbed liver function [1], leaving no other option than a liver transplantation. Although, it is still unknown which factors promote the development of NASH, new insights have revealed a role for factors derived from the adipose tissue commonly referred to as adipokines [2,3]. Adipokines have been shown to reach the liver upon secretion by the adipose tissue into the bloodstream. During obesity, an increased secretion of various pro-inflammatory adipokines has been reported, including tumor-necrosis factor (TNFα), interleukin 6 (IL-6) and interleukin 1β (IL-1β) [4]. As these are believed to activate inflammatory pathways in the liver [3], targeting of pro- or anti-inflammatory adipokines may hold great promise in preventing the development of NASH.

Recently, the novel adipokine chemerin has been implicated in the regulation of inflammation. Plasma chemerin levels have been demonstrated to correlate with many diseases associated with chronic inflammation, including Crohn’s disease, ulcerative colitis [5], chronic pancreatitis [6], atherosclerosis [7-11], type 2 diabetes [12-14] and NAFLD [13,15,16]. However, as these studies are based on a correlative assessment between chemerin and features of these diseases, the exact role of chemerin in the etiology of these diseases remains unclear. In addition, it is unknown if chemerin has a pro- or anti-inflammatory role [17] and thus may protect or accelerate inflammation. On the one hand, functional studies have shown that chemerin promotes inflammatory cell migration to sites of inflammation [18-21], and that chemerin signaling exacerbates central nervous system inflammation in a mouse model of autoimmune encephalomyelitis [22]. On the other hand co-administration of recombinant chemerin with lipopolysaccharide blunts the inflammatory response in a model of acute lung-inflammation [23]. In addition, pretreatment of peritoneal macrophages with chemerin inhibits the production of inflammatory mediators, while administering a neutralizing antibody against chemerin was shown to enhance the inflammatory response [24].

Consistent with this, controversial findings also exist in the presumed relationship between chemerin and NASH. We recently observed that expression of chemerin in visceral adipose tissue is negatively correlated with hepatic inflammation in patients
with NAFLD [16]. This suggests that chemerin may protect against the development of hepatic inflammation in NASH. However, in other studies a positive correlation was observed between plasma chemerin levels and markers of liver pathology in the obese population [13]. Furthermore, plasma chemerin levels in NASH patients were found to be elevated in comparison to patients with simple steatosis [15], suggesting that chemerin may aggravate the disease process. Adding to this controversy are the various expression levels of chemerin in the livers of NAFLD patients and in several mouse models of NAFLD, in which both increased and decreased chemerin expression levels have been observed [25-27].

Taken together, these studies indicate that chemerin is likely to be involved in the etiology of NASH, although it is still unclear whether it may attenuate or aggravate NASH. To determine if chemerin is causally involved in the etiology of NASH, functional studies using mouse models for NASH are essential. In a recent study, we investigated the role of the chemerin receptor, chemokine like receptor 1 (Cmklr1) in NASH [28]. In this study, whole body and hematopoietic Cmklr1 deficiency did not affect the development of hepatic steatosis or its progression to NASH. However, there are other chemerin receptors, Gpr1 and Ccr12 that may be able to compensate for the loss of Cmklr1 [19,29]. Although until recently no known function of these receptors was reported, a recent paper showed that Gpr1 is an active chemerin receptor that regulates glucose homeostasis [30]. Therefore, a role for chemerin in NASH cannot be completely determined by studying Cmklr1 deficient mice. To further elucidate if chemerin is causally involved in NASH, we overexpressed chemerin in the low-density lipoprotein receptor knock-out (Ldlr-/-) mouse model of NASH by using a recombinant self-complementary adeno-associated virus (scAAV) harboring full-length mouse chemerin.

**Material and methods**

**Mouse studies**

All experiments were performed according to Dutch law and approved by the Ethical Committee for Animal Experiments of the University of Groningen, the Netherlands. Experiments were carried out on female low-density lipoprotein receptor knock-out (Ldlr-/-) mice (8-12 weeks of age) bred inhouse and maintained on a 12-hour light/12-hour dark cycle with ad libitum access to food and water. Mice received a chow
diet or a high fat, high cholesterol diet containing 21% fat from milk butter and 0.2% cholesterol (HFC, Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France). Mice were kept on these diets for 12 weeks and an oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT) were performed following a 6 hour fast to assess insulin resistance. For the OGTT, a bolus of 2 g/kg glucose was given via oral gavage, whereas during the ITT, the mice received an intraperitoneal injection of insulin (0.3 U/kg). For both tests, blood glucose levels were measured using an OneTouch Ultra glucose meter before and at 15, 30, 60, 90 and 120 minutes after administration of the glucose and the insulin. Mice were sacrificed using cardiac puncture following a 6-hour fast and blood was collected for further analysis. Liver tissue was collected and snap-frozen in liquid nitrogen or fixed in 10% formalin. Paraffin-embedded liver sections (4 µm) were then stained with Hematoxylin-Eosin (HE) for histological analysis.

**Adeno-associated virus preparation**

To overexpress chemerin, a recombinant scAAV was created, either carrying full-length chemerin (chem-scAAV, see Table 1 for cDNA sequence) or no coding gene as a control (con-scAAV). The scAAV was created by transfecting HEK-293 A cells with an adenoviral plasmid, a helper plasmid and a construct with or without chemerin using polyethylenimine (PEI). The adenoviral plasmid and helper plasmid contained adenoviral helper functions E2A, E4 and Va, and the AAV genes Rep and Cap, respectively. The two constructs were under the control of the liver-specific LP-1 promotor and were flanked by AAV-ITRs to ensure packaging of the construct in the viral particle. Three days after transfection, the cells were lysed by three freeze/thaw cycles and the virus was isolated by iodixanol gradient centrifugation. The virus was purified using spin filters and titers were determined with real-time PCR using 2 sets of primers targeted against the inserted gene and the region overlapping the inserted gene and the backbone of the scAAV (Table 2). One week before the start of the HFC diet, mice were injected with 1x10^{11} viral particles per 20 g body weight in the orbital vein with either the chem-scAAV or the con-scAAV.
**TABLE 1.** cDNA sequence chemerin.

<table>
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<tr>
<th>cDNA</th>
<th>Sequence</th>
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</tr>
<tr>
<td></td>
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**TABLE 2.** Primer sequences virus titration.

<table>
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<th>Virus</th>
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<th>Reverse primer 5'-3'</th>
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</tr>
<tr>
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<td>Con-scAAV-2</td>
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<td>cagagggaggtgactgtt</td>
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**Cell culture studies**

Primary hepatocytes were isolated from male C57BL/6J mice following portal vein infusion with carbogenated Hanks I buffer for 10 minutes. After this, carbogenated Hanks II buffer with collagenase (84 U/ml) was infused for approximately 30 minutes. Liver was excised and hepatocytes were suspended in ice-cold Krebs buffer containing 0.01 g/ml BSA. Cells were washed and centrifuged and the pellet was resuspended in Williams E attach medium (containing 5% FBS, 0.5% Pen/strep, 50 um/ml Gentamycin, 0.25 ug/ml Fungizone and 0.2 ug/ml dexamethasone). Approximately 0.6 million cells/well were seeded in collagen I coated wells. Cells were allowed to attach for 4-5 hours, after which the medium was replaced by Williams E culture medium (containing 0.5% pen/strep, 50 ug/ml Gentamycin, 0.25 ug/ml Fungizone). The following day, cells were treated with either PBS or recombinant mouse chemerin (100 ng/ml, R&D Systems, MN)
for 4 hours and harvested using Qiazol reagent (Qiagen, Venlo, the Netherlands) for RNA isolation.

To isolate peritoneal macrophages, chow-fed C57BL/6 and Cmklr1−/− mice were sacrificed by cervical dislocation and injected with 3 ml ice-cold PBS (0.5% BSA) intraperitoneally. To dislodge attached macrophages, mice abdomens were massaged gently, after which PBS cell suspension was aspirated. Isolated cells were pelleted and resuspended in RPMI culture medium, containing 10% heat inactivated FCS and 1% Pen/Strep. Cells (0.5 million per dish) were seeded on a 12-well plate in 1 ml culture medium and incubated at 37 °C for 1 hour. Non-adherent cells were washed away with PBS and cells were incubated with fresh RPMI culture medium for 2 hours at 37 °C. Cells were washed with ice-cold PBS and harvested with Qiazol reagent to isolate RNA. Cell culture disposables were purchased from Greiner Bio-One (Alphen aan den Rijn, The Netherlands).

**Real-time PCR**

RNA was isolated using Qiazol reagent, according to the manufacturer’s instructions (Qiagen). cDNA was synthesized using the Transcriptor Universal cDNA Master kit from Roche, according to their instructions (Roche, Mannheim, Germany). Real-time (RT)-PCR was performed as described [31]. PCR disposables were purchased from Greiner Bio-One (Alphen aan den Rijn, The Netherlands). Primer sequences are listed in Table 3.

**TABLE 3. Primer sequences.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5’-3’</th>
<th>Reverse primer 5’-3’</th>
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<tr>
<td>Col1a1</td>
<td>aaccctgcggcgcaatg</td>
<td>cagacggtgcgtgaggggaac</td>
</tr>
</tbody>
</table>

**Plasma assays**

Plasma triglycerides, cholesterol and free fatty acids were determined at times of sacrifice using commercially available kits (cholesterol and triglycerides: Roche; free fatty acids: DiaSys, Holzheim, Germany). Plasma chemerin levels were determined at 2, 6 and 12
weeks after scAAV injection in mice fasted for 6 hours, using a commercially available ELISA kit (R&D systems, Abingdon, UK). Plasma insulin levels were measured after 6 hours of fasting using a commercially available ELISA kit (Alpco Diagnostics, Salem, NH) at the time of sacrifice.

**Lipid measurements**
To measure hepatic lipid content, lipids were extracted from crushed liver samples using Bligh and Dyer’s method [32]. Hepatic triglyceride and cholesterol levels were measured using kits that are commercially available (Roche, Mannheim, Germany).

**Statistical analysis**
Repeated measure ANOVAs, two-way ANOVAs or Mann-Whitney tests were performed using GraphPad Prism to test for the effects of diet and virus when appropriate. Bonferroni posttests were used to determine the effect of chemerin overexpression. P-values below 0.05 were considered significant and data are expressed as mean ± SEM.

**Results**

**Chemerin reduces inflammatory gene expression in primary mouse hepatocytes**
Given the controversial role of chemerin in inflammation and the primary role of macrophages in NASH, we first investigated whether a disruption in chemerin signaling, by ablation of **Cmklr1** may affect inflammatory gene expression in macrophages. Therefore, we isolated peritoneal macrophages from WT and **Cmklr1**⁻/⁻ mice. **Cmklr1**⁻/⁻ macrophages showed an increased expression of the inflammatory genes **Il-6**, **Tnfa** and **Il-1β** compared to WT macrophages (Fig. 1A), indicating that disrupted chemerin signaling results in a pro-inflammatory phenotype in macrophages. To investigate whether chemerin may inhibit inflammation in hepatocytes, isolated primary hepatocytes from C57BL/6 mice were stimulated with recombinant chemerin. As expected, the expression of **Il-1β** (Fig. 1B) and **Tnfa** (Fig. 1C) was reduced in hepatocytes stimulated with chemerin, indicating that chemerin has an anti-inflammatory effect on hepatocytes. Taken together, these results suggest that chemerin could protect against the development of hepatic inflammation in NASH.
Chemerin overexpression does not affect NASH

Figure 1. Chemerin signaling attenuates inflammation in vitro. (A) Basal inflammatory gene expression of interleukin 6 (Il-6), tumor necrosis factor α (Tnfα) and interleukin-1β (Il-1β) measured by real-time PCR in peritoneal macrophages isolated from C57BL/6 and chemokine-like receptor knock-out (Cmklr1-/-) mice. The average results from three independent experiments (each with an N = 3) are shown. Il-1β (B) and Tnfα (C) gene expression in primary mouse hepatocytes stimulated with PBS or recombinant mouse chemerin. Representative results from two independent experiments (N = 3) are shown. * P < 0.05 versus C57BL/6 peritoneal macrophages or PBS stimulated hepatocytes.

Hepatic overexpression of chemerin was maintained throughout the diet period

To investigate if chemerin alleviates NASH in vivo, we overexpressed chemerin in Ldlr-/- mice fed a chow diet or a high fat, high cholesterol (HFC) diet for 12 weeks using a self-complementary adeno-associated virus (scAAV) carrying full-length chemerin. To investigate if chemerin was successfully overexpressed in the livers of Ldlr-/- mice, we measured hepatic chemerin expression in mice sacrificed at the end of the 12-week diet period. On a chow diet, chemerin expression was 36-fold higher in mice that received the chemerin-scAAV (chem-scAAV) compared to controls (con-scAAV; Fig. 2A). On the HFC diet, chemerin expression was lower than in chem-scAAV mice on a chow diet, but still 22-fold higher compared to con-scAAV mice (Fig. 2A). In addition, we measured whether increased amounts of chemerin protein were secreted in the bloodstream of
chem-scAAV mice during the entire diet period. In both chow-fed (Fig. 2B) and HFC-fed (Fig. 2C) mice that received the chem-scAAV, plasma chemerin levels were significantly higher throughout the diet period. However, at the end of the diet period, plasma chemerin levels significantly declined to approximately 65% from the starting value in both dietary groups (Figs. 2B-C).

Figure 2. Hepatic chemerin overexpression in chow-fed and high fat, high cholesterol-fed Ldlr^{−/−} mice. Low-density lipoprotein receptor knock-out (Ldlr^{−/−}) mice were injected with a recombinant self-complementary adeno-associated virus carrying either no coding gene (con-scAAV) or chemerin (chem-scAAV) and fed a chow diet or a high fat, high cholesterol (HFC) diet for 12 weeks. (A) Chemerin gene expression was measured in the liver of con-scAAV and chem-scAAV mice sacrificed after 12 weeks of diet. Plasma chemerin levels in chow-fed (B) and HFC-fed (C) con-scAAV and chem-scAAV mice were measured at 2, 6 and 12 weeks during the 12-week diet period. N= 6-8 for all experiments. * P < 0.05 versus con-scAAV mice.

**FIGURE 2.** Hepatic chemerin overexpression in chow-fed and high fat, high cholesterol-fed Ldlr^{−/−} mice. Low-density lipoprotein receptor knock-out (Ldlr^{−/−}) mice were injected with a recombinant self-complementary adeno-associated virus carrying either no coding gene (con-scAAV) or chemerin (chem-scAAV) and fed a chow diet or a high fat, high cholesterol (HFC) diet for 12 weeks. (A) Chemerin gene expression was measured in the liver of con-scAAV and chem-scAAV mice sacrificed after 12 weeks of diet. Plasma chemerin levels in chow-fed (B) and HFC-fed (C) con-scAAV and chem-scAAV mice were measured at 2, 6 and 12 weeks during the 12-week diet period. N= 6-8 for all experiments. * P < 0.05 versus con-scAAV mice.

Hepatic overexpression of chemerin does not affect the development of NASH

Body weight was not affected by chemerin overexpression in mice fed a chow or HFC-diet (Fig. 3A). As expected, Ldlr^{−/−} mice developed dyslipidemia on a HFC-diet, as shown by an increased level of plasma triglycerides (Fig. 3B) and cholesterol (Fig. 3C). Although chemerin overexpression did not alter plasma triglyceride levels (Fig. 3B), plasma cholesterol levels were 15% lower in HFC-fed mice injected with the chem-
scAAV compared to controls (Fig. 3C). In the liver, chemerin overexpression did not alter liver weight, triglyceride or cholesterol levels in \(Ldlr^{-/-}\) mice fed either diet (Figs. 3D-F).

**FIGURE 3.** Hepatic chemerin overexpression does not affect body weight or hepatic lipid accumulation in \(Ldlr^{-/-}\) mice. Body weight (A), plasma triglycerides (B) and plasma cholesterol (C) were determined in low-density lipoprotein receptor knock-out (\(Ldlr^{-/-}\)) mice receiving the recombinant self-complementary adeno-associated virus carrying either no coding gene (con-scAAV) or chemerin (chem-scAAV) and fed with a chow or high fat, high cholesterol (HFC) diet for 12 weeks. (D) Liver weight, expressed as percentage of body weight, was measured at the time of sacrifice. Lipids were extracted from crushed liver samples and the amount of hepatic triglycerides (E) and hepatic cholesterol (F) was measured in chow-fed and HFC-fed con-scAAV and chem-scAAV mice. N= 8 for all experiments. * P < 0.05 versus chow-groups, \(^{\dagger}\) P < 0.05 versus con-scAAV mice.

To investigate whether chemerin overexpression protects \(Ldlr^{-/-}\) mice against the development of HFC-induced liver inflammation, we assessed the expression of several pro-inflammatory and pro-fibrotic genes. In line with previous observations [Gruben et al. chapter 2, 31,33], HFC-feeding increased the expression of pro-inflammatory (\(IL-1\beta\), \(Tnf\alpha\) and \(Cd68\)) and pro-fibrotic genes (\(\alpha Sm\) and \(ColIa1\)) compared to chow-fed mice (Figs. 4A-B). However, in contrast to our expectations, the expression of most of these genes was not affected by chemerin overexpression (Figs. 4A-B). Of note, \(IL-1\beta\) expression was moderately elevated, instead of reduced, in chem-scAAV mice compared to con-scAAV mice fed a HFC diet (Fig. 4A). Histological analysis of HE-stained liver sections confirmed the absence of major alterations in hepatic lipid accumulation or hepatic inflammation between the virus-groups (Fig. 4C).
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Figure 4. Hepatic chemerin overexpression does not attenuate hepatic inflammation in chow-fed and HFC-fed Ldlr⁻/⁻ mice. Ldlr⁻/⁻ mice injected with the self-complementary adeno-associated virus carrying no coding gene (con-scAAV) and the chemerin self-complementary adeno-associated virus (chem-scAAV) were fed a chow diet or a high fat, high cholesterol (HFC) diet for 12 weeks. (A) The expression of the pro-inflammatory genes interleukin-1β (Il-1β), tumor necrosis factor α (Tnfα) and Cd68 (A) and the profibrotic genes α smooth muscle actin (αSma) and collagen 1 alpha 1 (Col1a1) (B) was determined by real-time PCR and expressed as fold induction (N= 8). (C) Liver sections were stained with hematoxilin-eosin (HE) and representative pictures were taken (N = 6-8). The arrows indicate inflammatory foci. * P < 0.05 versus chow-groups, $P < 0.05$ versus con-scAAV mice.

Hepatic overexpression of chemerin does not affect the development of insulin resistance

Since chemerin has also been implicated in the development of insulin resistance and glucose intolerance [34-36], we next investigated the development of insulin resistance in Ldlr⁻/⁻ mice fed both chow and a HFC diet. Chemerin overexpression did not affect plasma glucose (Fig. 5A) or insulin (Fig. 5B) levels, nor was glucose (Figs. 5C-D) and insulin (Figs. 5E-F) tolerance affected by chemerin overexpression in both dietary groups.
Chemerin overexpression does not affect NASH

**Figure 5.** Hepatic chemerin overexpression does not alter glucose metabolism in chow-fed and HFC-fed Ldlr\(^{-/-}\) mice. Ldlr\(^{-/-}\) mice injected with a self-complementary adeno-associated virus carrying either no coding gene (con-scAAV) or chemerin (chem-scAAV) were fed a chow-diet or a high fat, high cholesterol (HFC) diet for 12 weeks. Plasma glucose (A) and insulin (B) levels were measured at the time of sacrifice (N = 8). A glucose tolerance test was performed in con-scAAV and chem-scAAV mice fed a chow diet (C) and a HFC diet (D) for 11 weeks (N = 7-8). An insulin tolerance test was performed by giving con-scAAV and chem-scAAV mice fed a chow diet (N = 5-6) (E) and a HFC diet (N = 8-9) (F) for 10 weeks an injection with 0.3 U/kg insulin.
Discussion

Despite the anti-inflammatory effects of chemerin in vitro, we found that chemerin overexpression in vivo did not reduce hepatic inflammation in Ldlr−/− mice. In addition, chemerin did not attenuate the accumulation of lipids or downregulate the expression of fibrosis genes, indicating that chemerin does not alleviate NASH in Ldlr−/− mice. These findings are in line with our previous publication, in which whole body as well as hematopoietic Cmklr1 deficiency in mice did not affect the development of NAFLD [28]. Whereas in the previous study, compensatory effects of the other chemerin receptors may explain the lack of an effect, we now show that also chemerin itself does not attenuate NASH.

Since neither hepatic chemerin overexpression, nor hematopoietic deletion of the chemerin receptor [28] affect NASH severity in Ldlr−/− mice, chemerin is probably not causally involved in its etiology. The correlations found between chemerin and NAFLD in humans and mouse models [13,15,26,37] are therefore probably not due to a causal relationship. Instead, these observations may result from a secondary effect of NAFLD [28]. Indeed, both chemerin and Cmklr1 levels are regulated by TNFα [38], FXR [25] and adiponectin [37], factors that all become deregulated in NAFLD. However, since we overexpressed chemerin in the liver, effects of chemerin expression in other tissues cannot be excluded by our study. Indeed, the effect of chemerin on NAFLD may also depend on its origin, as we observed in humans that only chemerin expression in visceral adipose tissue, and not in subcutaneous adipose tissue or the liver, was negatively correlated with hepatic inflammation [16]. To fully exclude a role for chemerin in the progression of NAFLD, tissue-specific chemerin knock-out mice should be investigated.

It is unclear what explains the discrepancies between the in vitro and in vivo data in our study. Although other authors have also shown that chemerin reduces inflammation in vitro [24], this was not confirmed by a later study [39]. Moreover, also in vivo studies have produced conflicting results [22,23], indicating that the role of chemerin in inflammation is complicated. It is possible that chemerin does reduce inflammation in hepatocytes in the liver, but that we cannot detect this because Kupffer cells are the main source of inflammation in the liver. As a result, the high level of inflammatory gene expression in Kupffer cells may mask chemerin’s effect on hepatocyte inflammation. To further elucidate this, Kupffer cells and hepatocytes should be individually analyzed. Another explanation for the discrepancies may lie in chemerin processing. Chemerin is processed by various proteases and different chemerin isoforms can be produced under
different conditions [40]. For the \textit{in vitro} studies, we administered an already active form of chemerin. For the \textit{in vivo} studies we overexpressed inactive, full-length chemerin, as this more closely resembles the physiological setting. The activation of chemerin in the \textit{in vivo} studies therefore depends on the presence of proteases in the body. In an early inflammatory state, mainly serine proteases may be secreted by neutrophils [41], which generate the most active chemerin peptides [40]. At a later stage, cysteine proteases may be secreted by macrophages [24], resulting in less active forms of chemerin [40]. Since inflammation is present for 12 weeks, the less active forms of chemerin may be produced. To investigate this, chemerin activity should be measured in chem-scAAV mice. It would also be interesting to measure which form of chemerin is present in human NAFLD, since up to now only total chemerin levels have been measured.

Plasma chemerin levels are also strongly associated with insulin resistance and type 2 diabetes in the obese population [12-14]. However, in contrast to previous publications that studied the function of chemerin in mouse models [34-36], we found no relation between chemerin and insulin resistance (Fig. 5). Becker \textit{et al.} found that overexpression of chemerin induced glucose intolerance and insulin resistance in skeletal muscle of \textit{Ldlr}\(^{-}\)\textit{mice} [35]. Similar to the current study, they kept the mice on a HFC diet (containing 0.21% cholesterol and 21% butterfat) for 12 weeks. However, in their study, human chemerin was used, whereas we used mouse chemerin. This may explain the discrepancies between the studies. Ernst \textit{et al.} found increased chemerin levels in mouse models of obesity and showed that administration of chemerin shortly before a glucose tolerance test exacerbated glucose intolerance in mice models of obesity and diabetes [34]. The differences with our study are likely to be explained by the acute administration of chemerin by Ernst \textit{et al.} in contrast with the chronic elevated chemerin levels in our study. Paradoxically, deficiency of chemerin or its receptor, \textit{Cmklr1}, also induced glucose intolerance [36,42], whereas we have previously shown that \textit{Cmklr1} deficiency does not affect glucose tolerance [28]. Together, these findings imply that the role of chemerin and \textit{Cmklr1} in the regulation of glucose metabolism is complex and more studies need to be dedicated to unravel these paradoxical findings.

An exciting observation in our study is that chemerin overexpression reduced plasma cholesterol levels by 15% on a HFC diet (Fig. 3C). This is clinically relevant as a 10% reduction in cholesterol corresponds to a 15% reduction in coronary heart disease mortality and an 11% decrease in total mortality [43]. As such, we expect to find a reduction in atherosclerotic lesion development following chemerin overexpression.
in $\text{Ldlr}^{-/-}$ mice fed a HFC diet, although this remains to be determined. Many studies have shown an association between plasma chemerin levels and cholesterol and/or atherosclerosis [7-11]. However, in contrast to our expectations, Becker et al. found that chemerin overexpression in $\text{Ldlr}^{-/-}$ mice did not affect plasma cholesterol levels or atherosclerosis [35]. Thus, the role of chemerin in atherosclerosis warrants further investigation.

The high chemerin expression achieved in this study, is a strong indication that chemerin is not involved in NASH. However, this high expression is also a limitation, because the levels are not physiological. Chemerin plasma levels were elevated approximately 10-fold, which is much higher than the 1.5 to 4-fold increase seen in human NAFLD [13,15]. Dose-response studies are necessary to determine if chemerin has an effect on NAFLD at physiological levels. Another limitation of our study is that feeding $\text{Ldlr}^{-/-}$ mice a HFC diet for 12 weeks did not induce hepatic steatosis as seen in other studies [28,33], although the diet did affect hepatic cholesterol accumulation and the expression of pro-inflammatory and fibrotic genes. The reasons for this are unclear, but may be attributed to the administration of the scAAV. Unfortunately, we cannot be certain, as we did not include mice without virus injection in our experiments. A lack of steatosis is not generally seen due to administration of a scAAV, so the effect observed here may be strain specific.

In summary, our data suggest that chemerin is not involved in the development of NAFLD or insulin resistance. Therefore, caution should be taken in interpreting the associations that are found between chemerin, NAFLD and insulin resistance [13,15].

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Chemerin overexpression does not affect NASH