Revisiting the roles of hepatic inflammation and adipokines in metabolic disease
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Chapter 8

General discussion
Chapter 8

Obesity in our time has taken on epidemic proportions and accounts for a high degree of morbidity. In particular, obesity is frequently accompanied by metabolic disease which leads in turn to cardiovascular complications. For this reason it is crucial to be able to treat the associated metabolic derangements such as insulin resistance and nonalcoholic fatty liver disease (NAFLD). Until now, however, treatment possibilities have been limited and can have detrimental side effects. Identification of novel mechanisms involved in the etiology of insulin resistance and NAFLD will contribute to the development of new, effective therapies. It was the aim of this thesis to elucidate the factors involved in the etiology of insulin resistance and NAFLD. To this end we investigated the role of hepatic inflammation and adipokines, and particularly chemerin. This chapter will present the major findings of the thesis, focusing on the multifactorial origin of NAFLD and type 2 diabetes (T2D) and addressing the clinical implications and future perspectives of this research.

NAFLD and T2D: multifactorial diseases

The etiology of NAFLD and T2D is complex. It cannot be explained by one single factor, as can monogenic disorders. This complexity becomes apparent when one studies the occurrence of these diseases in the obese population. Although NAFLD and T2D are clearly driven by obesity, one in four obese individuals is still metabolically healthy [1]. This suggests that there are other important factors. Besides alterations in systemic inflammation, tissue inflammation (e.g. liver, adipose tissue) and adipokine expression, mechanisms that have been implicated in the etiology of NAFLD and T2D include hereditary factors, environmental factors, the unfolded protein response, mitochondrial dysfunction and alterations in gut microbiota [2-4]. Ectopic lipid accumulation has also been considered to cause insulin resistance [5,6]. Therefore, hepatic lipid accumulation as seen in NAFLD may cause insulin resistance as well. On the other hand, insulin resistance also causes NAFLD. In chapter 7 we have discussed in detail this bidirectional role of NAFLD in insulin resistance.

Because of these multifactorial origins, animal studies investigating the etiology of T2D and NAFLD have been restricted to studying specific aspects of the disease. It is difficult, if not impossible, to develop a perfect disease model and no mouse model now available fully represents the metabolic derangements of an obese human [7-9]. For example, although C57BL/6 mice readily develop insulin resistance when fed a high fat
diet, they do not develop full-blown beta-cell failure [10,11]. Similarly, leptin deficient mice quickly develop hepatic steatosis through alterations in their hepatic lipid metabolism, but they do not spontaneously develop nonalcoholic steatohepatitis (NASH) [12]. For this a so-called second hit, for example in the form of endotoxins, is required [13]. Moreover, in humans these diseases take years to develop, whereas in mouse models this is simply not possible due to their short lifespan. Therefore, although a degree of simplification is necessary to identify the contribution of single factors and give valuable insights into the mechanisms involved, we must not forget that these diseases have complex origins. The results of such studies must be placed ‘in a more nuanced, relevant context’ [14]. This is crucial when interpreting studies investigating one single factor.

Hepatic inflammation and the multifactorial origins of insulin resistance

In this thesis, using two independent mouse models, the low-density lipoprotein receptor knock-out (Ldlr<sup>-/-</sup>) mice and mice expressing constitutively active inhibitor of κB-kinase-β (IKKβ) in hepatocytes (IKKβ<sub>ca</sub> hep mice) (chapters 2 and 3), we found that hepatic inflammation did not contribute to hepatic or systemic insulin resistance. However, although these and several other studies indicate that hepatic inflammation does not cause insulin resistance [15,16], we cannot disregard a number of important studies that did find a causal relationship [17-19]. As discussed in chapter 7, this controversy points to a more complex relationship between hepatic inflammation and insulin resistance.

In light of the multifactorial origins of insulin resistance, one could conclude that hepatic inflammation by itself is not enough, and that it contributes to insulin resistance only in combination or interaction with other factors. Since insulin resistance occurs mainly in the obese population, it is possible that hepatic inflammation causes insulin resistance only in the presence of obesity. This is supported by a study in which NF-κB activation was enhanced in mice by overexpression of P65. The resulting increased inflammation did not lead to insulin resistance, which was attributed instead to increased energy expenditure and inhibition of adipose tissue expansion [20]. On the other hand, Cai et al. did find a relationship between hepatic inflammation and insulin resistance, even though the mice were lean [17], suggesting that obesity is not a requirement for hepatic inflammation to cause insulin resistance. Another possibility is that hepatic inflammation contributes to insulin resistance only through crosstalk with
other organs. Cai’s study (2005) also showed systemic inflammation in IKKβcahep mice; they had increased circulating interleukin (IL)-6 levels and increased cytokine expression in the muscles [17]. Although we have not measured this in our IKKβcahep mice, in our Ldlr -/- mice hepatic inflammation did not induce adipose tissue inflammation (Chapter 2), suggesting that inflammation is contained in the liver. Moreover, in a previous study showing no relationship between hepatic inflammation and insulin resistance, systemic inflammation was not affected [15]; no alterations in adipose tissue inflammation or plasma cytokines were observed in this study (personal communication). Taken together, these studies suggest that only when associated with systemic effects does hepatic inflammation induce insulin resistance, whereas inflammation which is confined to the liver does not. Finally, it is also possible that hepatic inflammation plays no role at all in the development of insulin resistance. The association between hepatic inflammation and insulin resistance may simply be caused by a common underlying factor. This is not unlikely, as many factors are believed to affect both hepatic inflammation and insulin resistance, including alterations in the gut microbiota and hepatic lipid accumulation [2,21].

**Hepatic inflammation and insulin resistance in humans**

In obesity, the plasma levels of many adipokines and cytokines are altered and associated with insulin resistance [22,23]. In humans the inflammatory markers in plasma are strongly associated with the level of adipose tissue inflammation [24], indicating that adipose tissue is at least partially responsible for systemic inflammation. However, many of these cytokines and adipokines can also be produced by the liver itself [23,25,26]. Unfortunately the contribution of the liver to the alterations in plasma levels of adipokines and cytokines in humans is not known. Of rodents it is known that obesity leads to activation of NF-κB in the liver [17]. Moreover, liver inflammation in mice also increases circulating cytokines, including IL-6 [17]. This has led to the idea that hepatic inflammation may also contribute to the low-grade chronic inflammatory state and development of insulin resistance in obesity. However, in this thesis we dispute this idea. What is the evidence from humans that hepatic inflammation contributes to insulin resistance?

NASH is characterized by hepatic inflammation and is in humans closely linked to insulin resistance [27]. However, since NASH usually occurs only with obesity, other obesity-associated changes, and not hepatic inflammation, may be the cause of the
insulin resistance. This is supported by the lack of insulin resistance due to hepatic inflammation in lean mouse models as reported in this thesis (chapters 2 and 3). In addition, hepatic inflammation was shown to lead to steatosis [28-31], which in turn could cause insulin resistance [5]. However, this relationship remains controversial (chapter 7).

The relationship between NASH and insulin resistance could also be explained by the fact that insulin resistance leads to enhanced hepatic lipid accumulation which also causes liver inflammation (chapter 7).

Other factors that cause hepatic inflammation may provide further evidence. Hepatitis C virus (HCV) infection leads to chronic inflammation like that observed in NASH. Patients with HCV have an increased number of macrophages and an increased pro-inflammatory gene expression in the liver [32]. In support of a role for HCV infection in insulin resistance, several studies did find an association between the two [33-36]. This association could be caused by the inflammation found in HCV patients, since pro-inflammatory cytokines were correlated with insulin resistance [37]. However, another study showed no such association [38]. Moreover, a recently published study disputes the association between HCV infection and insulin resistance [39]. In this study insulin resistance was correlated mainly with the degree of liver damage, as measured by ALT and AST, and not with HCV infection. Together, these studies indicate that in humans the role of hepatic inflammation in insulin resistance is far from clear.

The link between inflammation and insulin resistance revisited

Since this thesis questions the role of hepatic inflammation in the development of insulin resistance, is it also possible that low-grade chronic inflammation in general is not involved in its pathogenesis? Although many studies support a role for inflammation in insulin resistance, there is evidence that questions this role. First of all, in humans there is much genetic variation in inflammatory genes. However, most genes identified through genome-wide association studies to be associated with T2D are involved in beta-cell function and do not play a role in inflammation [40]. Variations in inflammatory genes are associated mainly with inflammatory diseases like inflammatory bowel disease and auto-immune diseases [41,42]. This does not support a role for inflammation in T2D. Nonetheless, since much of the genetic susceptibility to T2D remains to be explained [40], it is possible that inflammatory genes associated with T2D will be discovered in the future. At present, extensive studies investigating the relationship between inflammatory
genes and insulin resistance are lacking. Interestingly, though, a recently published paper identified CD44 expression in adipose tissue as a candidate gene for T2D [43].

Secondly, in studying the role of inflammation in T2D, mostly knock-out mice models of inflammatory genes were used. Yet, while knock-out models are a useful tool, this may overestimate the role of inflammation because these genes may not be involved solely in inflammation. This is illustrated by mice deficient in the pro-inflammatory cytokine IL-18. These mice present with hyperphagia, resulting in adiposity and insulin resistance. This was prevented by administering IL-18 intracerebrally, indicating that IL-18 is not involved only in inflammation, but also in the regulation of food intake [44].

Furthermore, adipose tissue inflammation in mice does not necessarily lead to insulin resistance. For example, increased adipose tissue inflammation, induced by adipocyte-specific expression of constitutively active IKKβ, has been reported to protect against hepatic and systemic insulin resistance [45]. This was attributed to their increased energy expenditure and reduced adipose tissue [45]. Moreover, mice with increased systemic and adipose tissue inflammation due to expression of transmembrane tumor necrosis factor (Tnf)α have local insulin resistance, but also have improved systemic insulin sensitivity [20]. Therefore, adipose tissue inflammation does not necessarily induce systemic insulin resistance. To the contrary, reduced adipose tissue inflammation was recently shown to induce insulin resistance [46]. This paper showed that adipose tissue inflammation is necessary for healthy adipose tissue expansion. Suppression of adipose tissue inflammation by expression of a dominant-negative form of Tnfα specifically in adipose tissue led to increased metabolic dysfunction, including insulin resistance and NAFLD. Surprisingly, like the models with increased adipose tissue inflammation, these mice also had reduced adipose tissue mass. This indicates that the increased insulin resistance found in the study cannot be attributed to changes in adiposity but is associated with increased systemic inflammation, hepatic steatosis and decreased intestinal barrier function [46]. Taken together, these studies suggest that inflammation in itself does not necessarily lead to insulin resistance.
General discussion

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Box 1. The importance of ‘negative’ data

In this thesis we found that hepatic inflammation does not affect insulin resistance in **Ldlr**−/− mice (**chapter 2**) and **IKKβca**hop mice (**chapter 3**). In addition, we found that chemerin and its receptor are not involved in the development of NAFLD (**chapters 5 and 6**). These so-called ‘negative’ findings are important, because in order for science to progress it is necessary to determine the factors that are not involved. As Edison said: ‘I can never find the thing that does the job best until I find the ones that don’t.’ Moreover, publishing negative data gives a more objective overview of all the data available. When negative results are not published, the positive effects are overestimated and experiments that fail are needlessly repeated. This costs society a lot of time and money and possibly increases the use of laboratory animals. Unfortunately, studies in which no significant differences are found are often considered less interesting than studies with significant differences. That negative data are believed to be less exciting is strange, because if an experiment is well-designed it could be considered to be more reliable than positive data [47,48].

Our ‘negative’ results point to a more complex relationship between inflammation and insulin resistance than previously believed. This is further supported by the lack of efficacy of anti-inflammatory drugs. ‘Indeed, until a specific anti-inflammatory agent shows robust efficacy in humans, the role of tissue inflammation in causing insulin resistance in obese and T2DM subjects remains incompletely established’ [2]. Moreover, our results indicate that other mechanisms are likely to be involved in the development of insulin resistance. We also showed that the association between chemerin and NAFLD is probably not a causal relationship, but may be due merely to other factors that are dysregulated in NAFLD. This is important to know in order to give a reliable overview of the possible effectiveness of drugs targeting chemerin. Stimulating the publication of negative data will benefit the scientific community as well as society because it will prevent waste of time and money and bring available data closer to the truth. Therefore, I recommend that more effort be made to publish negative results.

**Adipokines and the multifactorial origin of T2D and NAFLD**

We found a clear association between the expression of **leptin**, **chemerin**, **ANGPT2**, **TNFα**, **CXCL10**, **IGF1**, **IL1RN** and **PAI-1** and features of NAFLD (**chapter 4**), suggesting that these adipokines may be involved in the development of some of these features. Given the functions of these adipokines (mainly in inflammation, discussed in detail in **chapter 4**), it is not surprising that they could be involved in the etiology of NAFLD. Therefore their role in the etiology of NAFLD deserves further investigation. In this thesis, however, we focused on chemerin (**chapters 5 and 6**), because of the strong negative association between chemerin expression in visceral adipose tissue and lobular inflammation in the liver.

Knockdown of the chemerin receptor and overexpression of chemerin in the liver did not affect NAFLD in mice. This suggests that the association found between chemerin
expression and NAFLD or insulin resistance is not causal. Nevertheless, some limitations must be taken into account when interpreting these results. First, since these knockdown and overexpression experiments were performed on mice, we cannot fully exclude a causal relationship in humans. However, it is likely that chemerin has a similar function in mice and humans, as both share similarities in chemerin expression. In several mouse models of NAFLD, hepatic chemerin expression was decreased; this was in the same study also observed in humans with NAFLD [49]. Additionally, the amino acid sequence is well conserved in both mice and humans [50].

A second limitation is that we studied the role of chemerin and its receptor only in NAFLD and insulin resistance, whereas it is well-documented that in obesity adipose tissue is dysfunctional and that the expression of many adipokines becomes dysregulated [22,23]. These adipokines exert different effects on metabolism and inflammation, and it is probably the combination of changes in adipokines that drives the development of disease. In the case of progression from hepatic steatosis towards NASH it is likely that multiple hits are necessary [21]. We took this into account in chapters 5 and 6 by giving the mice a diet with a high fat content, mimicking the full-spectrum of changes that occur in diet-induced obesity in humans. However, in Ldlr<sup>−/−</sup> mice receiving the adeno-associated virus expressing chemerin, no differences in body weight were observed when feeding the mice a high-fat diet. Since these mice probably did not have dysfunctional adipose tissue, they were possibly not as susceptible to metabolic disease as obese mice. We therefore suggest that the role of chemerin in metabolic disease be tested further in models more metabolically challenged, such as Ob/ob mice or Db/db mice.

**Treatment**

**Treatment with anti-inflammatory drugs**

The results reported in this thesis raise the question as to whether anti-inflammatory drugs, especially if targeted at the liver, are a good strategy for the treatment of insulin resistance. Indeed, anti-inflammatory therapies have not been altogether successful. The most effective anti-inflammatory agents used to treat insulin resistance are salicylates. Salicylates improve insulin resistance in obese mice and patients with T2D [51-53]. More recently, the one-year safety of salicylates was evaluated in a large randomized clinical trial [54]. In this study, when compared to a placebo, salicylates maintained a glucose-lowering
effect in patients, and decreased their Hb1ac levels by 0.37%. This result was associated with a decrease in inflammatory markers in plasma. Alterations in liver inflammation were not reported but body weight, plasma lipid levels and urinary albumin levels increased. Therefore, the long-term cardiorenal safety requires further study.

Resveratrol is a polyphenol already available as a nutrient supplement. Because of its anti-inflammatory properties this supplement has become of interest as a medicine for metabolic syndrome. Resveratrol decreases NF-kB activity in vitro by inhibiting the expression of the enzymes cyclooxygenase 2 and prostaglandin E synthase-1 [55,56]. These enzymes are involved in the production of pro-inflammatory mediators. In rhesus monkeys, resveratrol was shown to reduce inflammation and insulin resistance. However, in human trials the effect of resveratrol on inflammation has been controversial. Several studies reported significant anti-inflammatory effects [57-59], whereas others did not [60-63]. Also, some studies reported an effect of resveratrol on insulin resistance [57-60], but not all [61-63]. Moreover, effects on insulin resistance are not always accompanied by a reduction in inflammation [60], suggesting that resveratrol improves insulin resistance via other mechanisms.

Other therapies directed at more specific inflammatory mediators are also not greatly successful. Blocking TNFα improved insulin resistance in several [64,65] but certainly not all studies [66-68]. Blocking IL-1β has been shown to improve plasma glucose levels, but not by enhancing insulin sensitivity; the effects were attributed to enhanced beta-cell function [69,70]. The low effectiveness of anti-inflammatory drugs has been attributed to their inability to lower inflammation in the adipose tissue [2]. Whether these drugs also inhibit inflammation in the liver is unknown. Further research into the roles of hepatic and adipose tissue inflammation must include investigation of the effect of tissue specific treatment of inflammation.

**Treatment with adipokines**

Adipokines are involved in the regulation of lipid metabolism, inflammation and glucose homeostasis and are therefore interesting therapeutic targets for the metabolic syndrome [22,23]. In mouse models the beneficial effects of many adipokines have been shown. For example, administration of adiponectin to mouse models of the metabolic syndrome enhances insulin sensitivity and reduces hyperglycemia and steatosis [71,72]. In addition, features of the metabolic syndrome are improved in mice by administration of FGF21 [73,74]. Although the therapeutic potential of these adipokines has not been investigated
directly in clinical trials there is evidence that several commonly used therapies for the metabolic syndrome act through activation of adipokines. Pioglitazone exerts its positive effects on insulin resistance and NAFLD at least partly through adiponectin [75].

In this thesis we showed that the association between chemerin and lobular inflammation in humans (chapter 4) is probably not a causal one (chapters 5 and 6). Therefore therapies directed against chemerin may not be beneficial for NAFLD. In addition, this thesis also questions a role for chemerin as a therapeutic target for insulin resistance (chapters 5 and 6). We cannot, however, exclude a therapeutic potential for chemerin in other inflammatory diseases; this requires further investigation. The functional role and therapeutic potential of the other adipokines (leptin, ANGPT2, TNFa, CXCL10, IGF1, IL1RN and PAI-1) found in this thesis to be associated with NAFLD also require further investigation.

Future perspectives

What is chronic low-grade inflammation?
One of the questions that remain is how the hepatic inflammation achieved in our models relates to chronic low-grade inflammation in human obesity, for which there is no exact definition. In obese individuals the level of inflammatory cytokines is only twofold higher than in non-obese subjects [76,77]. In obese mice the increase or decrease of inflammatory gene expression in tissues is often much higher or lower compared to lean mice, which may therefore not be representative of what happens in human obesity. High levels of inflammation may even have other strong interfering effects on metabolism, as for example a catabolic response [20,45]. Therefore, more subtle mouse models are required, such as gain-of-function models and tissue-specific models. The gain-of-function models represent more closely what happens in obesity, as they show an increase in low-grade inflammation. Tissue-specific models are useful for investigating the roles of different organs. However, when using these models one must always keep in mind that other organs may also be affected because of crosstalk between tissues. In addition, techniques now available make it possible to study genes that are partially knocked out, the so-called hypomorphic models. These models are less likely to overestimate an effect. It would also be of interest to investigate the role of inflammation in different strains of mice. For metabolic disease, mainly C57BL/6 mice
are used because these mice have a strong innate immune response. The use of other strains with a less developed innate immune response may provide answers as to how important inflammation really is. Finally, the effects of the duration of inflammation should be investigated in more longitudinal studies.

**Gut microbiota and the metabolic syndrome**

Since we have indicated that hepatic inflammation does not contribute to insulin resistance, other possible causes of insulin resistance and NAFLD should be further investigated. Recently, for example, alterations in the gut microbiota in obese individuals have received a lot of attention. These alterations in gut microbiota occur through overnutrition and differences in dietary intake [78,79]. There is a strong correlation between alterations in gut microbiota and metabolic disease. Current reports also show that alterations caused by the intake of artificial sweeteners induce glucose intolerance [80]. In addition, NAFLD patients were shown to have bacterial overgrowth [81], whereas T2D patients were shown to have fewer butyrate producing bacteria [82]. Butyrate and other short chain fatty acids are believed to protect the host against the adverse effects of gut microbiota by preventing a ‘leaky gut’. In a leaky gut, endotoxins from gut microbiota leak into the circulation and cause endotoxemia and systemic inflammation. Patients with the metabolic syndrome were shown to suffer from endotoxemia [83,84]. This suggests that alterations in gut microbiota may be the origin of the chronic low-grade inflammation found in obesity [85]. That gut microbiota likely plays a causal role in metabolic disease was shown when feces of lean individuals transplanted into obese individuals significantly improved insulin resistance [86]. However, this was shown only for particular feces donors and the number of participants in this study was small. Taken together, there is much evidence to suggest that gut microbiota plays a role in metabolic disease. However, it remains to be determined if and which particular bacterial species are involved and could be used as therapeutic targets. In addition, more research is needed to establish the most effective way to influence the microbiota.

**MicroRNAs and the metabolic syndrome**

microRNAs (miRNAs) have recently been identified as key regulators of metabolic homeostasis [87] and immunity [88]. miRNAs are small non-coding RNAs of 19-25 nucleotides that regulate gene expression at the RNA level. They bind to target mRNAs by sequence complementarity and inhibit protein synthesis by translational repression of
mRNAs or by promoting mRNA degradation [89]. Evidence is accumulating to support a role for miRNAs in metabolic disease. For example, miRNA-34a was upregulated in a mouse model of NAFLD [90] and high levels of miRNA-34a are associated with NAFLD and NASH in human patients [91,92]. These effects may be due to inhibition of SIRT1 expression in the liver by miRNA-34a [93], as SIRT1 is known to regulate many genes involved in lipid metabolism and inflammation such as SREBPs, PPARα, PGC1α, LXR, FOXO, and NF-κB [87]. SIRT1 activators, such as resveratrol, have previously been shown to improve hepatic steatosis and insulin resistance in obese mice [94]. The therapeutic potential of miRNA inhibition in liver disease was demonstrated in a clinical trial in which antisense inhibition of miRNA-122 was used to treat chronic hepatitis C [95]. This points to miRNAs as interesting new therapeutic targets for metabolic disease, targets which deserve further investigation.

Healthy versus unhealthy obese individuals
Another important tool to find the causes of insulin resistance and NAFLD is to study the differences between metabolically healthy and unhealthy obese individuals. A recent study showed that heme-oxygenase (HO)-1 is necessary for the development of low-grade chronic inflammation and metabolic disease [96]. In this study, unhealthy obese individuals had higher HO-1 protein levels than healthy obese individuals. Moreover, HO-1 levels correlated strongly with metabolic disease. This may explain why some people are obese and metabolically healthy and others are not. The critical role of HO-1 in metabolic disease was confirmed by hepatocyte- and macrophage-specific knock-out models which were both protected from metabolic disease. This was due to a reduction in the inflammatory response. Conversely, overexpression of HO-1 induced metabolic disease. This study nicely illustrates what can be learned from studying differences between unhealthy and healthy obese individuals. In addition, some lean individuals that are metabolically unhealthy could also be used as a tool to identify causes [97].

Concluding remarks
To avert the T2D and NAFLD epidemic, it is important to identify the mechanisms involved in their development. Since this thesis questions the role of hepatic inflammation and chemerin in insulin resistance and NAFLD, and anti-inflammatory therapies have not as yet been overly successful, it is of interest to explore other possible mechanisms such as the role of microbiota and microRNAs. This may lead to the development of more effective drugs.
References


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Chapter 8


