



Universiteit
Leiden
The Netherlands

Hepatic steatosis : metabolic consequences

Boer, A.M. den

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Chapter 8

General Discussion and Future Perspectives

In this thesis the metabolic causes and consequences of hepatic steatosis are described. Hepatic steatosis is characterized by excessive accumulation of triglycerides (TG). The prevalence of hepatic steatosis will certainly increase in the near future, associated with the expected exponential increase in the prevalence of obesity and type 2 diabetes mellitus.¹⁻³ At present, hepatic steatosis is observed already in 3-24 % of healthy subjects and even in 84-96 % of morbidly obese subjects.⁴ Hepatic steatosis was considered a benign, histological condition, until it was discovered that a fatty liver is associated with cardiovascular risk factors such as increased levels of VLDL-TG, glucose, PAI and fibrinogen.⁵ Because the liver is the central organ in the disturbances in glucose and lipid metabolism, the main questions are: Are these associations links in a chain or spokes on a wheel and what could then be the common feature or cause connecting the spokes? Although many studies have shown strong associations between hepatic TG content and hepatic insulin resistance^{6,7}, only few studies have investigated the mechanisms underlying this association. We consider fatty liver as a mediator in the perturbations of glucose and lipid metabolism. Hepatic steatosis can be both actively and passively involved in these metabolic disturbances.

Comments on the measurements of hepatic insulin sensitivity

A glucose tolerance test can not discriminate between whole-body and liver-specific insulin sensitivity. Therefore, in our studies we used the golden standard for measuring whole body and liver-specific insulin sensitivity: the hyperinsulinemic euglycemic clamp technique. By primed continuous infusion of D-[3-³H]glucose and the measurement of the specific activity of this tracer, we can discriminate between the amount of glucose produced by the liver and the amount of glucose taken up by peripheral tissues. In Chapter 3 we have compared the dose-dependent effects of insulin on glucose production and VLDL-TG production by the liver under hyperinsulinemic euglycemic conditions with different insulin concentrations. Interestingly, although the liver plays a central role in both glucose and lipid metabolism, these two processes are differentially regulated by insulin. We found that hepatic glucose output (HGO) is much more sensitive to insulin-mediated inhibition than hepatic VLDL-TG production. The mechanism behind this difference in insulin sensitivity remains unclear.

The mammalian body, especially the brain, largely depends on glucose as an energy substrate. From a teleological perspective it is tempting to speculate that maybe therefore, plasma glucose levels are tightly regulated, even after a carbohydrate containing meal. In contrast, after a fat containing meal, a large increase in plasma fatty acids (FA) and TG can be observed. This may be due to the fact that the hepatic VLDL-TG production is less sensitive to insulin-mediated inhibition than HGO. Normally insulin-mediated suppression of HGO is used as a measure of hepatic insulin sensitivity, but it is also relevant to consider insulin sensitivity of hepatic VLDL-TG production. It appears that these two processes do not change in parallel. For instance, we found in our CD36-deficient mice that although HGO is severely insulin resistant, the hepatic VLDL-TG production was not different under hyperinsulinemic conditions between *cd36*^{-/-} mice and control littermates (83 ± 2 vs 94 ± 3 $\mu\text{mol TG/kg bodyweight/h}$; unpublished observations). It would be interesting to determine whether this dissociation between insulin sensitivity of HGO and of hepatic VLDL-TG production also occurs in other conditions.

The amount of insulin that is infused and the resulting plasma insulin levels are of major importance for the implementation and interpretation of the hyperinsulinemic euglycemic clamp analysis. A low insulin dose already suppresses HGO, whereas no effect on hepatic VLDL-TG production may be observed. Infusion of high insulin dosages may lead to the overlooking of subtle differences in hepatic insulin sensitivity, especially with regard to HGO. In the ideal situation plasma insulin levels are always similar in experimental groups to allow comparison of the clamp results. For different reasons, however, the resulting plasma insulin levels sometimes differ between groups, despite the infusion of identical amounts of insulin. Some studies correct for plasma insulin levels in their results, but should this be allowed? In Chapter 5 we also found a difference in plasma insulin levels between groups, despite the infusion of identical amounts of insulin. We decided not to correct for this observation, since we do not know the underlying cause of this difference in plasma insulin levels. Insulin can be cleared faster, with or without having an impact on insulin signaling. Therefore, we suggest that when the cause and/or consequence of different plasma insulin levels is not clear, corrections should not be used.

Another important aspect, that needs to be considered in the design of hyperinsulinemic euglycemic clamp experiments, is the use of anesthetics. In this thesis all clamp studies are performed in mice anaesthetized with acetylpromazine,

midazolam and fentanyl (VDF). Early on in our studies we were forced to switch from one combination of anesthetics to another combination for practical considerations, i.e. the availability of the anesthetics. To validate the new anesthetics we compared the old regimen (fluanisone, midazolam and fentanyl; HM) with two new combinations: VDF versus medetomidine, midazolam and fentanyl (MMF) on parameters obtained during clamp experiments. We found that MMF caused severe insulin resistance, whereas HM and VDF did not affect insulin sensitivity. Therefore, it is of great importance to validate anesthetics in all physiological experiments, to exclude possible interference of these drugs with normal metabolism.

Hepatic steatosis with hepatic insulin resistance

In this thesis we have used several murine models with targeted disruptions of the FA metabolism. The *cd36*^{-/-} mice and the ritonavir- (RTV-)treated mice confirm the inverse association between increased liver lipid content and decreased hepatic insulin sensitivity. In these two models we investigated the mechanisms behind the disturbances in the lipid metabolism, leading to increased plasma FA and TG levels.

CD36-deficient mice

CD36, or fatty acid translocase (FAT), is involved in the high affinity uptake of FA in the periphery. Mice lacking CD36 have considerably impaired FA uptake in muscle and in adipose tissue.⁸ These mice exhibit increased plasma FA and TG levels and show decreased plasma glucose levels.⁹ In the liver plasma membrane FA-binding protein (FABPpm), but not CD36, is the main FA transporter.¹⁰ Consequently, in *cd36*^{-/-} mice the increased plasma FA level leads to increased uptake of FA by the liver. This increased flux of FA leads to an increase in β -oxidation, reflected in increased plasma levels of ketone bodies. The increased FA flux, however, largely exceeds β -oxidation capacity. These excess FA, that cannot be oxidized, are stored as TG and steatosis develops. Previously, Goudriaan *et al.* showed that *cd36*^{-/-} mice exhibit hepatic steatosis and severely decreased hepatic insulin sensitivity.¹¹ If the liver would have been able to increase the production of VLDL-TG, this increase of hepatic TG content could have been prevented. We showed in Chapter 4 that the increased plasma TG levels in CD36 deficiency were not due to a previously hypothesized enhancing effect on hepatic VLDL-TG production or an effect on intestinal lipid absorption. Instead, CD36 deficiency caused hypertriglyceridemia by

decreased LPL-mediated hydrolysis of TG-rich lipoproteins resulting from FA-induced product inhibition.

Increased plasma FA levels are commonly associated with insulin resistance.¹² In the *cd36^{-/-}* mice despite increased plasma FA (and TG) levels, the periphery is even more sensitive to insulin-stimulated glucose uptake compared to controls.¹¹ It appears that tissue-specific uptake of FA is more important than plasma FA levels *per se*. The *cd36^{-/-}* mice may be more sensitive to insulin-stimulation of glucose uptake, because in the periphery there is no possibility to use FA as an energy source. This is in accordance with the Randle hypothesis, which states that the availability of FA for oxidation determines insulin sensitivity and the rate of glucose oxidation.^{13,14}

Ritonavir-treated mice

The introduction of highly active antiviral therapy (HAART) has led to a considerable reduction in the morbidity and mortality that was associated with HIV-infection. Unfortunately, these drugs are associated with severe adverse metabolic effects, such as the lipodystrophy syndrome. In this syndrome subcutaneous wasting of fat is observed (lipoatrophy) with or without accumulation of fat in the dorso-cervical region (“buffalo hump”) or in the abdomen (lipodystrophy). Several metabolic disturbances such as hyperlipidemia, hyperglycemia and insulin resistance are observed in subjects with the lipodystrophy syndrome. Hepatic steatosis is also observed frequently.¹⁵ Few studies have shown a direct mechanism involved in the emergence of this syndrome. Several studies indicated that the hyperlipidemia induced by HIV protease inhibitors such as RTV is due to an increase in hepatic VLDL-TG production. A study in HIV-infected patients hypothesized that excessive FA mobilization occurred due to insulin resistance of adipose tissue resulting in increased hepatic VLDL-TG production.¹⁶ Studies in C57Bl/6 and AKR/J mice showed increased VLDL-TG production after RTV treatment.^{17,18} Evidence also existed that HIV protease inhibitors do not reduce the clearance of VLDL-TG particles¹⁷⁻¹⁹ providing additional support for a mechanism based on increased production of TG-rich particles. However, other studies indicated that impaired lipoprotein clearance may contribute to protease inhibitor-induced hyperlipidemia. Baril *et al.* found that both LPL and hepatic lipase (HL) were decreased in HIV-infected patients treated with protease inhibitors such as RTV.²⁰ TG-rich lipoprotein

clearance was reduced in HIV-patients after a high fat meal.²¹ Obviously, many contradictory hypotheses with regard to the mechanism underlying protease inhibitor-induced hyperlipidemia existed. In Chapter 6 we conclusively elucidated the mechanism behind RTV-induced hypertriglyceridemia. RTV decreases plasma LPL activity, either by decreasing expression levels of LPL but most probably also via inhibition of the activity of the LPL enzyme that is present. With respect to the underlying mechanism of lipodystrophy, we found that the adipose tissue of RTV-treated mice takes up less FA derived from the plasma free FA pool and from VLDL-TG particles. Therefore, long-term inhibition of FA uptake by adipose tissue may eventually lead to decreased adipose tissue mass. In addition, we found in unpublished observations that RTV-treated mice showed hepatic steatosis and hepatic insulin resistance (Figure 1A and 1B). It may be that the excess FA that cannot be taken up into the adipose tissue are taken up by the liver, although this was not evident from the data of our study on tissue-specific FA uptake. There is an intriguing resemblance between the *cd36*^{-/-} mice described in Chapter 4 and RTV treated mice (Chapter 6). Apparently, in both mouse models hypertriglyceridemia is present and FA uptake from plasma is decreased. RTV-treated and CD36 deficient mice show hepatic steatosis and severe hepatic insulin resistance as is shown in Figure 1. However, in *cd36*^{-/-} mice this is associated with increased peripheral insulin sensitivity, whereas in RTV-treated mice peripheral insulin sensitivity was not changed. Most likely, this discrepancy indicates that there are different tissue specific alterations between both models, which were not addressed directly in our study design. For instance, muscle TG content was increased in RTV-treated mice compared to control mice, whereas it remained unchanged in *cd36*^{-/-} mice compared to littermates.

Interestingly, in presence of excess adipose tissue (obesity) and in the absence of adipose tissue (lipoatrophy), similar metabolic disturbances are observed: hyperglycemia, hyperinsulinemia and hyperlipidemia. Disturbances in adipose tissue metabolism affect hepatic FA/TG metabolism, and *vice versa*. Several important questions remain, however. At present, it remains unclear to what extent the results obtained in the RTV-treated APOE*3-Leiden transgenic mice can be extended to the action of protease inhibitors in HIV-infected patients. In addition, it is important to understand the actual biochemical mechanism(s) behind the RTV-induced decrease in adipose tissue FA uptake. For instance, the selectivity for adipose tissue suggests

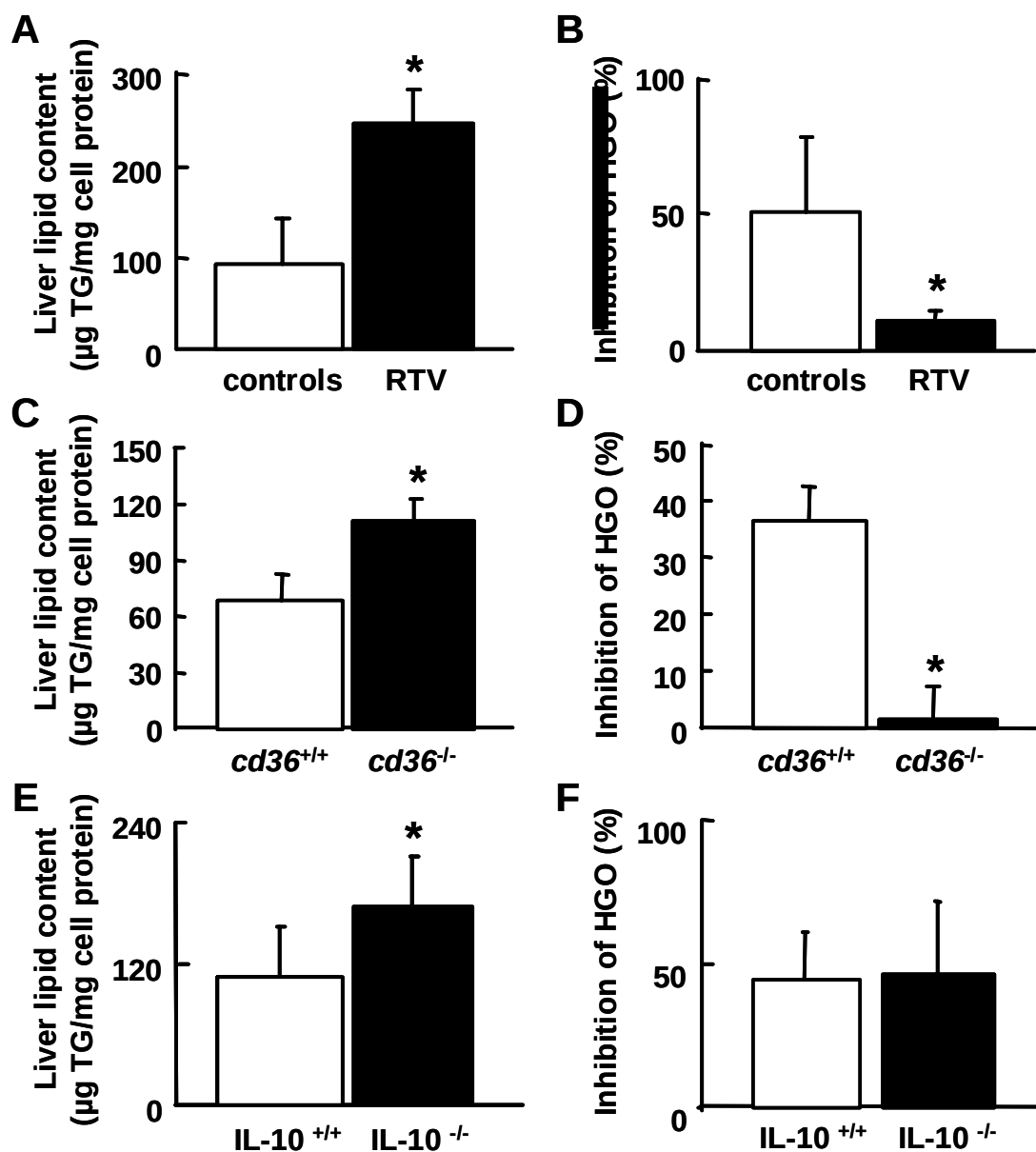


Figure 1. Hepatic TG content and insulin sensitivity in the 3 described models.

Using high performance thin layer chromatography hepatic TG content was determined in RTV-treated mice (A), *cd36*^{-/-} mice (C) and *IL-10*^{-/-} mice (E) and their appropriate controls. Hepatic insulin sensitivity was determined using the hyperinsulinemic euglycemic clamp analysis. RTV-treated mice (B) and *cd36*^{-/-} mice (D) showed a significantly decreased insulin-mediated inhibition of hepatic glucose output whereas in *IL-10*^{-/-} mice (F) hepatic insulin sensitivity remained unchanged. * $P < 0.05$

the possible involvement of factors like peroxisome proliferator-activated receptor γ (PPAR γ) which is also important in the regulation of CD36. Further studies are needed to investigate the molecular mechanism behind the lipodystrophy syndrome. It could be speculated that HIV-infected patients have a high risk of developing hepatic steatosis. Multiple factors have been hypothesized to be necessary for the development and progression of this condition.²² Potential risk factors in HIV-infected individuals include disturbances in glucose and lipid metabolism, chronic inflammation, hepatitis co-infection, and treatment with antiretroviral drugs such as protease inhibitors. Hepatic steatosis, which is often observed in HIV-infected subjects, is associated with increased plasma glucose, FA and TG levels which are traditional cardiovascular risk factors. However, studies on steatosis in HIV-infected patients are still rare. Nevertheless, while waiting for prospective studies in HIV-infected patients, improved recognition, diagnosis and management of steatosis are required in these patients.

Hepatic steatosis and atherosclerotic risk

Human cohort studies showed that HAART-treated patients are at greater risk of developing premature atherosclerosis.²³ This group however has increased cardiovascular risk factors which may overshadow the beneficial inhibitory effects of RTV on atherosclerosis.²⁴ HIV-infected patients with CHD are older than patients without CHD.²³ Patients may already have some atherosclerotic lesion formation due to their age, whereas our mice started treatment while they were “young adults”. Several opportunistic infections may play a role in the pathophysiology of CHD. It has been suggested that cytomegalovirus and *Chlamydiae pneumoniae* may promote atherosclerosis.²⁵ Furthermore, before treatment is started patients have been exposed to chronic systemic inflammation due to HIV-infection for sometimes up to 10 years. The prevalence of the traditional risk factor cigarette smoking is high among HIV-infected patients with CHD (69 %).²³ It may be of interest to follow HIV-infected children on HAART, and follow the development of atherosclerosis in these subjects. The problem here is that it will probably take up to 50 years before conclusive results can be drawn from such a study. Therefore, in this thesis we studied the development of atherosclerosis in RTV treated mice, which developed hepatic steatosis and hepatic insulin resistance, in addition to an atherogenic lipoprotein profile. From these adverse effects of RTV on cardiovascular risk factors,

we expected that RTV would induce or accelerate atherosclerosis. However, in contrast to our expectations, RTV protects against the development of atherosclerosis in the APOE*3-Leiden transgenic mice (Chapter 7). The important question is to what extent we can extrapolate this remarkable observation in (APOE*3-Leiden transgenic) mice to RTV-treated HIV-infected humans, treated with other HAART drugs as well. Nonetheless, at present, our mouse model is the most appropriate substitute, in which we can study the effects of drugs such as RTV without the many complicating genetic and environmental factors that can influence results in human studies.

In the literature discussion exists whether hepatic steatosis should be added to a cluster of cardiovascular risk factors (metabolic syndrome) important in determining cardiovascular risk. Since a fatty liver is involved the production of cardiovascular risk factors, it may be important to take this condition into consideration when establishing individual cardiovascular risk. However, the fact that the relationship between hepatic steatosis and metabolic disturbances leading to increased cardiovascular risk is apparently not straightforward has to be taken into account.

Hepatic steatosis without hepatic insulin resistance

The association between increased hepatic TG content and hepatic insulin resistance does not always hold. In Chapter 2 we already discussed some dissociations in this respect, for example the *ob/ob* mouse treated with rosiglitazone²⁶ or wild type mice treated with LXR-agonists.²⁷ These models show increased hepatic TG content with paradoxically increased or unchanged hepatic insulin sensitivity compared to their respective controls. Another mouse model with increased hepatic TG content without a change in hepatic insulin sensitivity is the interleukin-10-(IL-10-) deficient mouse.

IL-10 deficient mice

In epidemiological studies insulin resistance is associated with chronic low-grade inflammation.²⁸ This is reflected in associations between the degree of insulin sensitivity and plasma levels of several cytokines, such as tumor necrosis factor (TNF) α and interleukin-(IL)6.^{29,30} IL-10 is a potent anti-inflammatory cytokine, which is produced by T-cells, B-cells, monocytes and macrophages and plays a crucial role in the innate immune system.^{31,32} IL-10 potently inhibits the production of pro-inflammatory cytokines, including TNF α and IL-6.³³ Previous studies in humans have

shown an association between the production capacity of IL-10 by blood cells and cardiovascular risk factors.³⁴ To evaluate a causal relationship between IL-10 production and metabolic dysregulation, we assessed in Chapter 5 the direct consequences of IL-10 deficiency on hepatic and peripheral insulin sensitivity. Our data showed, that basal IL-10 production protects against hepatic steatosis during high fat feeding (Figure 1E). However, endogenous IL-10 production did not improve hepatic or whole-body insulin sensitivity during high fat feeding as assessed by the hyperinsulinemic euglycemic clamp technique (Figure 1F). This finding is in contrast to the strong association that is found between liver TG content and insulin resistance in several other models (Chapter 2). Strikingly, the IL-10^{-/-} mice showed decreased plasma insulin levels compared to control mice while infusing similar insulin concentrations. Although this complicates the interpretation of the clamp results, we can still conclude, that basal IL-10 expression does not improve hepatic insulin sensitivity. It would be interesting to perform insulin clearance studies in these mice to gain a better insight into the mechanism behind this difference in plasma insulin levels.

The cause of the increased liver TG content may be the increased plasma FA levels after overnight fasting. The increased plasma FA levels are most probably due to the increased visceral fat mass in the IL-10^{-/-} mice compared to their wild type counterparts. Interleukins have been shown to affect adipose tissue metabolism in other murine models. The IL-1 receptor antagonist knockout (IL-1Ra^{-/-}) mice have a defect in lipid accumulation in adipose tissue, exhibiting leanness, which could be expected from their catabolic state.³⁵ IL-6-deficient mice developed obesity and obesity-related disorders which could be partly reversed by replacement with the pro-inflammatory IL-6.³⁶ The absence of the anti-inflammatory IL-10 was also expected to lead to a higher inflammatory (catabolic) state, and consequently, to a decreased amount of adipose tissue. However, plasma levels of fibrinogen and serum amyloid A, which reflect liver and systemic inflammation, respectively, were not changed in the IL-10^{-/-} mice compared to the wild type controls. In contrast to our expectations, we found an increased amount of visceral adipose tissue in the IL-10^{-/-} mice compared to the wild type controls. We currently do not know why the adipose tissue mass is increased in the IL-10-deficient mice. A factor that largely determines the uptake of FA by the adipose tissue is LPL-activity. An oral fat load experiment in which plasma TG and FA appearance in time are measured after an oral olive oil

bolus may give an indication of LPL-activity in the IL-10^{-/-} mice. It is also interesting to measure the uptake of FA by the adipose tissue in these mice to determine whether there is an increased FA uptake from VLDL-TG or the albumin-bound FA pool leading to increased adipose tissue mass.³⁷ In our clamp study we did not determine adipose tissue-specific insulin sensitivity. The ~40% decrease in plasma FA during hyperinsulinemia in both the IL-10^{-/-} mice and the control mice suggests no change in adipose tissue insulin sensitivity. To exclude an effect of IL-10 deficiency on adipose tissue insulin sensitivity more specific *in vivo* and *in vitro* experiments investigating adipose tissue lipolysis are required.

Our observations in the IL-10-deficient mice argue against a simple protective role of endogenous IL-10 secretion in insulin resistant states. Nonetheless, our data also indicate that endogenous IL-10 secretion is not metabolically inert, since we documented clear effects of IL-10 deficiency on hepatic and peripheral lipid metabolism. However, our study did not support a causal role of IL-10 in the protection against diet-induced hepatic insulin resistance and other metabolic disturbances. IL-10 is a locally acting cytokine, and therefore plasma levels may not be causally involved in insulin resistance. The results from epidemiological studies investigating similar plasma parameters should therefore be interpreted with caution with respect to underlying causal mechanisms.

Hepatic steatosis: Cause or consequence of metabolic disturbances?

The different models used in this thesis clearly show, that not every form of hepatic steatosis has the same metabolic causes and consequences. Different causes of steatosis may have different metabolic effects. Human studies investigating causes of fatty liver and consequent metabolic disturbances showed that etiology can make a difference.³⁸ Like in several mouse models, the causes and effects of hepatic steatosis in humans probably also depend on the genetic and environmental background. This remains difficult to investigate this since the liver is not easily accessible in humans. Therefore, we decided to study the causes and consequences of hepatic steatosis in several mouse models.

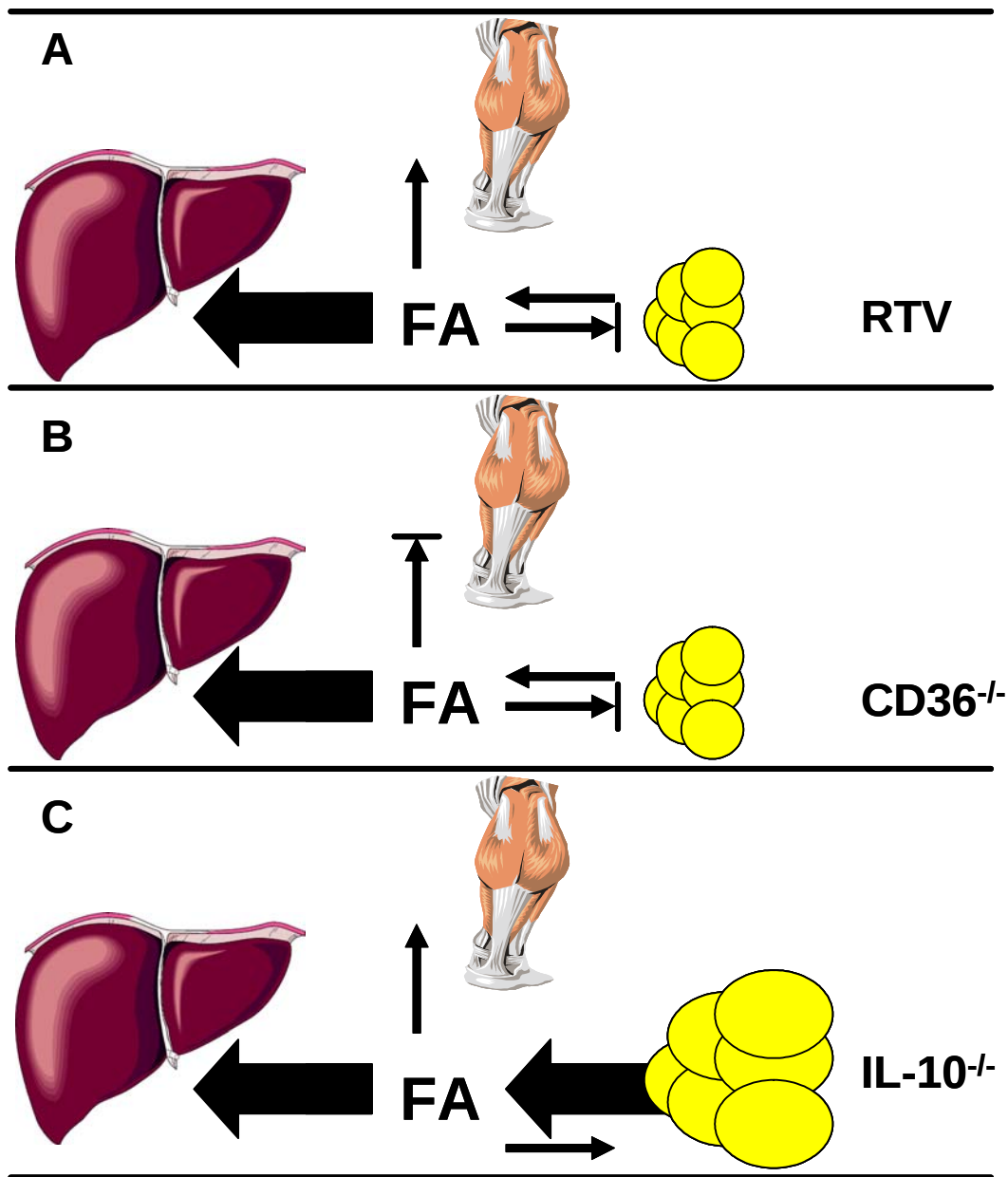


Figure 2. Increased plasma FA fluxes cause hepatic steatosis. The causes and consequences of hepatic steatosis differ between the three models described in this thesis, but in all models an increased flux of FA is most probably involved. **A.** RTV-treated mice show increased plasma FA levels which are due to decreased FA uptake by adipose tissue and an increased postprandial FA response. **B.** CD36-deficient mice have increased plasma FA levels due to decreased uptake of FA in peripheral tissues such as adipose tissue and muscle. **C.** IL-10-deficient mice show increased plasma FA levels after overnight fasting which are most probably due to the increased visceral adipose tissue mass observed in these mice.

Plasma FA flux appears to be important in the emergence of a fatty liver. The 3 models studied in this thesis all show increased plasma FA levels which are due to decreased FA uptake and/or decreased LPL-mediated TG hydrolysis or increased FA release from adipose tissue as is shown in Figure 2. The *cd36*^{-/-} mice and the IL-10^{-/-} mice both show hepatic steatosis, most probably due to increased plasma FA levels. This induces hepatic insulin resistance in the *cd36*^{-/-} mice, but not in the IL-10^{-/-} mice. Both mouse models show increased plasma FA levels after overnight fasting. In the *cd36*^{-/-} mice this is due to decreased peripheral FA uptake.⁸ In the IL-10^{-/-} mice this is probably due to an increased release of FA from the increased visceral adipose tissue mass (Chapter 5). The important difference between these two models is the exposure time to the increased plasma FA. The *cd36*^{-/-} mice have increased plasma FA levels from birth, or even *in utero*, while the IL-10^{-/-} mice only displayed increased plasma FA after overnight fasting. The RTV-treated mice show hepatic steatosis and hepatic insulin resistance (Figure 1A and B), but here the cause is unclear. It has been hypothesized that RTV induces accumulation of activated forms of sterol regulatory binding protein (SREBP)-1 and -2 in the nucleus of liver and adipose tissue, resulting in elevated expression of lipid metabolism genes.³⁹ We observed that postprandially these mice show significantly increased plasma TG and FA, but the plasma FA and TG levels were also increased after 4 h fasting. Similar to the *cd36*^{-/-} mice, the RTV-treated mice may also be continuously exposed to increased plasma FA levels which may be involved in the emergence of steatosis and insulin resistance.

We have not investigated the distribution of the TG in the hepatic lobules by histology. This may also be of importance in the different metabolic causes and consequences of hepatic steatosis since metabolic pathways are not uniformly distributed in the liver.⁴⁰⁻⁴² Diabetes-associated steatosis is predominantly present in the perivenous zones of the liver.

In recent studies from the group of Rossetti the role of the brain in the regulation of insulin action on the liver was investigated.⁴³⁻⁴⁵ The overall conclusion from those studies was that insulin-mediated control of HGO is controlled by the brain, and more specifically, by the hypothalamus. No studies have yet been performed on the role of the brain in other aspects of hepatic insulin sensitivity such as in the control of hepatic VLDL-TG production. It would be interesting to investigate this aspect of insulin action on the liver in a model without hypothalamic control. The hepatic

glucose production is under parasympathetic and sympathetic neuronal control, which can be eliminated in an experimental setting by transection of the hepatic parasympathetic or sympathetic nerves.⁴⁶ With these studies of Rossetti in mind, it would be interesting to determine to what extent hypothalamic control is involved in different consequences of hepatic steatosis. *Cd36*^{-/-} mice especially would lend themselves as good models to investigate this aspect of insulin sensitivity.

In this thesis we considered the causes and consequences of hepatic steatosis. The liver is an essential organ involved in the integrative physiology of whole-body glucose and FA metabolism. It is very difficult to dissect the causes and consequences of hepatic steatosis in the intact individual, due to the complex interactions between different organs. These interactions include multiple metabolic and endocrine factors transported by the blood between organs and also tissue-specific activity of the autonomous nervous system. The hierarchy between these different factors in modulating hepatic insulin sensitivity remains at present unclear. In general, experimental conditions are usually focused on a single factor. Therefore, the relative contribution of each of these individual factors on the metabolic causes and effects of liver steatosis is difficult to estimate. Because the prevalence of metabolic syndrome is reaching endemic proportions, it is important to investigate the causes and consequences of hepatic steatosis both in human and in animal studies.

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