Universiteit
Leiden
The Netherlands

Modulation of VLDL triglyceride metabolism

Bijland, S.

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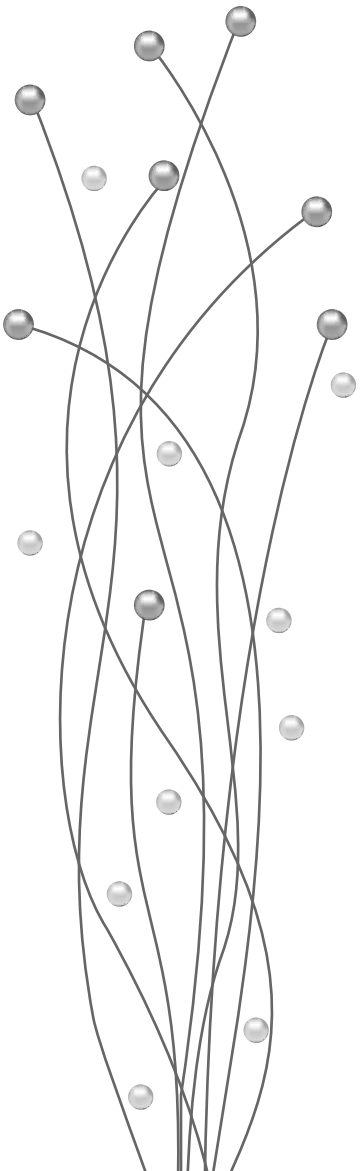
Chapter 7

General discussion



Nowadays, our energy intake chronically exceeds the energy we use, and therefore fat accumulates resulting in overweight and obesity. The energy in our diet is stored as carbohydrates and fat. Since the storage of carbohydrates in the body is limited, excess energy is stored as fat and mobilized again during fasting to provide energy. This mobilization occurs by the release of fatty acids (FA) by adipose tissue into the circulation. Part of the FA are used directly for ATP supply following oxidation, but a large fraction of the FA enters the liver where FA are re-esterified into triglycerides (TG) that are secreted as part of the core of VLDL particles by the liver. These VLDL particles deliver FA to peripheral tissues where they can be used to generate energy. The cycling of FA and TG enables us to adapt to changes in energy demand, but the western daily energy intake exceeds our ability to maintain homeostasis.

Obesity is strongly associated with metabolic dyslipidemia, which is characterized by low levels of HDL-cholesterol (C) and high levels of TG. Additional pathologies associated with obesity include high blood pressure and systemic inflammation and, collectively, these abnormalities are known as the metabolic syndrome (MetS). MetS is probably the most prevalent cause for the current increase in patients with diabetes mellitus type 2 (DM2) and cardiovascular disease (CVD). The studies described in this thesis evaluated several aspects of the regulation of VLDL-TG metabolism and the main conclusion and implications of our findings are discussed in this chapter.

CETP and VLDL metabolism

Cholesteryl ester transfer protein (CETP) transfers TG and cholesteryl esters between lipoproteins and has a major impact on cholesterol metabolism by decreasing HDL-C and increasing LDL-C. Although CETP also transfers TG, it was not clear whether and to what extent CETP would affect TG metabolism. In a small study with three heterozygous CETP deficient persons, it was shown that the postprandial TG response after an oral fat load was decreased compared to normal persons, suggesting that CETP may retard TG clearance from plasma[250]. In naturally CETP-deficient mice, it was shown that expression of human CETP increased the postprandial TG response after an oral fat load

by reducing the clearance rate of chylomicron-like lipoprotein particles, due to reduced LPL activity[140]. Thus, CETP may have the capacity to affect TG metabolism.

In **chapter 2**, we studied the role of CETP on VLDL metabolism in ApoE*3-Leiden (E3L) mice. E3L mice have a more human-like VLDL metabolism than wild type mice and have been extensively used to dissect the effects of diets and drugs on lipoprotein metabolism[114, 115, 117, 118]. The expression of human CETP, under control of its natural flanking regions, in the E3L background enables us to study both VLDL and HDL metabolism in a more human-like setting. Our experiments in **chapter 2** indicate that CETP mainly affects cholesterol metabolism and not TG metabolism. In addition, expression of CETP did not affect high fat diet-induced obesity. This suggests that also under conditions where TG/FA metabolism is stressed, CETP does not affect TG metabolism. The major implication of these findings is that it is unlikely that pharmaceutical modulation of CETP activity affects TG metabolism and obesity in humans. Although this does require validation in human studies, it indicates that potential negative side-effects of CETP inhibitors in relation to obesity and related diseases are less likely to occur.

Recently, several drugs have been developed to inhibit CETP, thereby increasing HDL-C and reducing the risk for CVD. Of these CETP inhibitors, torcetrapib was the first drug used in large clinical trials (RADIANCE[251], ILLUSTRATE[252], and ILLUMINATE[122]). Although the outcome of these trials were negative, probably related to off-target side-effects of torcetrapib, combination therapy of atorvastatin and torcetrapib for 2 years increased HDL-C by almost 60% compared to patients receiving atorvastatin alone. In these studies, torcetrapib caused only a minor reduction in plasma TG (-16%). Short-term treatment with anacetrapib, a more recent CETP inhibitor, had no effect on plasma TG levels[253]. These data support our findings that CETP inhibition does not evoke major effects of plasma TG metabolism.

Our data also indicate that CETP does not affect the development of diet induced obesity. However, obese subjects do present with increased circulating levels of CETP, indicating that obesity per se does affect CETP level and activity. It is likely that this is a direct consequence of the increase in adipose tissue, which has been demonstrated to be an important source of plasma CETP[254, 255, 256].

This is further supported by the reduction in CETP mass and activity after marked weight loss[257, 258, 259]. However, since obesity is associated with multiple metabolic abnormalities, it cannot be excluded that alternative mechanisms explain the association of obesity with increased CETP activity.

Plasma CETP activity is dependent on CETP levels, protein levels of activators and inhibitors, as well as on the presence and composition of donor and acceptor particles for lipid exchange. Variations in the CETP gene, characterized by single point mutations (called single nucleotide polymorphisms, SNPs), are related to changes in CETP plasma levels and activity. The most studied SNP in the CETP gene is the so-called Taq1B polymorphism. CETP mass and activity are higher in carriers of the Taq1B1 allele compared to carriers of the Taq1B2 allele, which is associated with lower HDL-C levels in B1 carriers[260]. Most meta-analyses show that high TG levels are causal to the interaction of the Taq1B polymorphism with HDL-C in obese subjects by providing more acceptors for the transfer of cholesterol from HDL to apoB containing lipoproteins[261]. Low levels of HDL-C and high levels of TG are risk factors for the development of CVD. Therefore it appears that although CETP does not directly affect TG metabolism, TG levels do determine the effect of genetic variation in CETP on cholesterol metabolism thereby increasing the risk to develop CVD.

Nuclear receptor ligands and VLDL metabolism

Nuclear receptors play an important role in the orchestration of energy metabolism and are important targets for drug development for diseases associated with the MetS. Nuclear receptors regulate the expression of genes involved in energy homeostasis and numerous other processes by binding to specific DNA regulatory elements. The natural ligands of nuclear receptors include hormones and lipid intermediates. One of the physiological roles of a subset of nuclear receptors is to function as sensors of energy status by activating or inhibiting specific target genes and pathways to maintain homeostasis.

Various drugs act by functioning as synthetic ligands for specific nuclear receptors and thus affect energy metabolism. For example, the fibrate class of

compounds activate peroxisome proliferator-activated receptor alpha (PPARα) and are prescribed to lower plasma TG levels. Thiazolidinediones (TZDs) activate PPARγ and are prescribed to lower plasma glucose and lipid levels in DM2 patients. However, TZDs also lower FA turnover, thereby effectively lowering hepatic fat content[262]. More drugs that target nuclear receptors do also influence lipid and energy metabolism as a side effect. For example, rifampicin is an antibiotic drug that activates pregnane X receptor (PXR) and can cause hepatic lipid accumulation and steatosis[86, 180, 181]. In this thesis, a number of compounds that act via nuclear receptors have been investigated to determine the precise mechanism by which they affect VLDL-TG metabolism.

Fenofibrate

In **chapter 3** we focussed on the effect of the TG-lowering drug and PPARα activator fenofibrate on VLDL-TG metabolism. In the past, it has been shown that activation of PPARα lowers plasma TG levels by increasing the clearance of VLDL-TG. However, it was also suggested that PPARα reduces VLDL-TG production. First, in vitro experiments showed that incubation of cultured hepatocytes with fenofibrate reduced TG secretion[153]. Second, experiments using the strong PPARα agonists Wy14643 also showed a reduction of VLDL secretion in vivo[152]. In addition, mice lacking PPARα show increased VLDL production[150, 151]. However, the effect of fibrates on VLDL secretion in humans remains inconclusive as either decreased or no changes in apoB production were observed whereas no effects on VLDL-TG production have been reported[263, 149].

To dissect the effect of PPARα modulation on VLDL-TG metabolism in a more human-like setting, we used E3L.CETP mice receiving a clinically relevant dose of fenofibrate. In **chapter 3** we showed that fenofibrate actually increases rather than decreases VLDL-TG secretion without altering the rate of VLDL-apoB production. Furthermore, VLDL-TG clearance was strongly enhanced explaining the overall reduction in plasma TG, which can be explained by an increase in of lipoprotein lipase (LPL) activity. Part of the FA liberated by lipolysis are not directly taken up by the underlying tissue but add to the plasma pool of albumin-bound FA, which is taken up by the liver. The increased hepatic uptake of albumin-bound FA contributed to the increased

flux of FA through the liver, resulting in higher VLDL-TG production and an increased particle size of nascent VLDL particles. This implies that PPARα activation by fenofibrate increases FA turnover.

In MetS, and especially obesity, FA overflow also occurs and it is believed that the increased FA turnover plays a role in the development of DM2. However, treatment with fenofibrate in diet-induced obese mice showed that activation of PPARα actually prevented severe obesity, adipocyte hypertrophy and maintained normal glycaemia[264, 265]. Together these data suggest that the increased FA turnover per se is not sufficient to induce pathology associated with MetS and is likely compensated by the regulation of additional pathways affected by PPARα activation.

Systemic inflammation is another feature of MetS that might trigger pathology in obese people. PPARs have been shown to play a role in macrophage activation in the vasculature thereby reducing atherosclerosis[266]. PPARα has been shown to have a more general effect on inflammation by inhibiting the activation of peritoneal macrophages by lipopolysaccharide (LPS)[267]. Activation of PPARα inhibits the expression of genes involved in the acute phase response and inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α)[268]. Patients with dyslipidemia indeed show reduced levels of IL-6 and TNF-α when treated with fenofibrate[269]. Therefore the suppression of inflammation might be the reason why increased FA turnover by fenofibrate does not induce pathology. This is supported by a study in mice showing that hepatic PPARα activation suppresses expression of hepatic TNF-α and reduces hepatic fat accumulation during the development of fatty liver disease[270]. Small studies in humans showed no reduction of hepatic TG content upon treatment with fenofibrate[263, 271]. However in one of these studies, patients with NAFLD treated with fenofibrate did show improvement of MetS and liver function[271]. It would therefore be interesting to determine whether this anti-inflammatory effect of PPARα activation is sufficient to prevent pathology associated with increased FA turnover.

Since side-effects have been reported for fenofibrate including gastrointestinal disorders and skin reactions, new PPARα therapeutics are being developed that exhibit tissue- and gene-selective activities. Collectively, such agonists are known as selective PPAR modulators (SPPARMs). These

SSPARMs may provide novel insight in the role of specific PPARα-induced pathways in specific tissues in the development of MetS.

Rifampicin

Rifampicin is an antibiotic prescribed for the treatment of tuberculosis and is also a ligand for the nuclear receptor pregnane X receptor (PXR). Activation of PXR is associated with alterations in lipid metabolism and hepatic steatosis[85, 86, 180, 181, 182, 272]. Furthermore, rifampicin might also directly affect lipoprotein metabolism since its metabolites (known as ansamycins) can directly bind lipoproteins which might influence lipoprotein clearance[273, 274]. In **chapter 4** we determined the effect of rifampicin on VLDL-TG metabolism. Treatment with rifampicin induced hepatic steatosis and strongly reduced plasma cholesterol levels whereas plasma TG levels were unaltered. The reduction of cholesterol was mainly confined to the VLDL-sized fraction and due to reduced VLDL-particle production by the liver. However, this was not reflected by a reduction in plasma TG levels. This could be explained by TG enrichment of secreted VLDL itself.

Rifampicin treatment in obese people should be critically considered. In obesity, insulin resistance of the liver and adipose tissues is associated with hypertriglyceridemia due to increased VLDL-TG production. This can be attributed to the failure of insulin to suppress the FA flux from adipose tissue to the liver and the failure of insulin to inhibit VLDL production. Increased levels of VLDL-TG result in increased CETP activity and reduced HDL-C levels. Treating obese patients with rifampicin might further reduce HDL-C levels in a CETP-independent manner by reducing the formation of HDL. Overall this may further increase their risk to develop CVD.

Liver insulin resistance in obesity is also associated with hepatic steatosis. Similar to our findings in mice, rifampicin treatment has been reported to cause hepatic steatosis, especially in patients that consumed large amounts of alcohol. People consuming more than 6 alcoholic drinks per day are at great risk to develop alcoholic hepatic steatosis since alcohol affects multiple steps in hepatic FA metabolism. Thus, care should be taken in the administration of rifampicin to heavy drinkers as well as patients with the MetS, since this might exacerbate the exisiting hepatic steatosis.

Perfluoroalkyl sulfonates

Chemical pollutants such as pesticides, industrial solvents and plasticizers can disrupt energy homeostasis by interfering with nuclear receptor-mediated gene expression. We are exposed to these pollutants via water, air and our diet and they mimic lipids or hormones due to their chemical structure. However, how and to what extent FA/TG metabolism is affected by these pollutants is hardly known.

Perfluoroalkyl sulfonates (PFAS) are widely used as repellents, surfactants and fire-retardant foams[195, 199]. They are extremely resistant to metabolic and environmental degradation and therefore bio-accumulate. Studies in animal models have shown that PFAS affect plasma lipid parameters although the exact mechanism is not known. In **chapter 5**, we show that PFAS activate multiple nuclear receptors and mainly PPARα and the xenobiotic receptors Constitutive Androstane Receptor (CAR) and PXR. This results in an almost complete blockade of both VLDL-TG and VLDL-apoB production, whereas VLDL-TG clearance is increased.

In addition to PFAS, there are various chemical pollutants present in our environment that can affect energy homeostasis of which some have recently been shown to cause obesity (called obesogens). Obesogens can be functionally defined as chemical agents that promote lipid accumulation and adipogenesis[275, 276]. These obesogens include bisphenol A, various xenoestrogens, organotins and phthalates, many of which end up in the human due to their use as, for example, surfactants or due to the ubiquitous use of pesticides and plastics from which they are derived. In addition to influencing energy homeostasis by activating multiple nuclear receptors, these pollutants might also affect inflammatory gene expression via the same nuclear receptors. However, the net effect of these compounds on the various pathways that are regulated via nuclear receptors are largely unexplored.

Role of nuclear receptors in metabolic stress

Whole body energy homeostasis is tightly regulated. During fasting or excessive caloric intake, energy balance is disrupted and adaptations in energy metabolism are necessary to maintain homeostasis. Both PPARα and CAR

are involved in the metabolic adaptations that occur during fasting. PPARα regulates the uptake and transport of FA in the cell, activates β-oxidation, and activates ketone body synthesis. CAR on the other hand inhibits β-oxidation by competing with PPARα for its binding site in the 3-hydroxyacyl-CoA dehydrogenase gene promoter, an important enzyme of peroxisomal FA β-oxidation. CAR also inhibits gluconeogenesis[79, 277]. There are reports indicating that PPARα ligands such as Wy14643 and ciprofibrate induce CAR expression[278, 279]. However, activation of CAR was not seen in our fenofibrate study in **chapter 3**. It is more than likely that under physiological conditions subtle changes in the activation of multiple nuclear receptors regulate the response of TG and energy metabolism. It is interesting to study if these nuclear receptor mediated changes in energy homeostasis are associated with pathology. We therefore compared the effect of fasting and high fat diet feeding on the expression of genes involved in lipid metabolism in the liver as shown in **chapter 6**.

Since the liver plays an important role in adjusting lipid and energy homeostasis, we investigated the changes in liver gene expression after prolonged fasting and 2 weeks of high fat feeding in mice. One of the major findings in **chapter 6** is the level of gene regulation involved in the adaptation of energy homeostasis. During fasting, over 400 genes were differently expressed compared to control, whereas during high fat feeding, only 65 genes were differently expressed compared to control. This suggests a very complex transcriptional regulation of energy homeostasis during fasting

Both during fasting and high fat feeding, the liver switches to lipid metabolism thereby increasing β-oxidation. One of the major differences between fasting and high fat feeding includes the up-regulation of genes involved in FA biosynthesis upon high fat feeding, while these genes are down-regulated after prolonged fasting. During both fasting and high fat feeding, the liver becomes steatotic. However, only after high fat feeding the accumulation of liver lipid is associated with the development of insulin resistance[230]. The origin of this difference may reside in the fate of the TG pool in response to fasting, which is oxidation. Whereas the fate of the TG pool in response to high fat feeding is ectopic storage. The exact mechanism involved in the development of insulin resistance in response to high fat feeding requires

further investigation.

It is believed that metabolic inflexibility plays a role in the pathology of the MetS. Both during fasting but also in obesity and insulin resistance, FA oxidation increases[280, 281, 282, 283]. However, when glucose becomes available again, for example during re-feeding, the body switches from FA to glucose oxidation. In obese and DM2 subjects switching between fat and glucose oxidation is impaired, a phenomenon described as metabolic inflexibility[284, 285]. PPARα plays a dominant role in lipid oxidation and therefore likely plays a key role in metabolic flexibility[286].

However, it has become apparent that in obese subjects lipogenesis is activated in addition to increased β-oxidation by PPARα activation. This increased lipogenesis has been attributed to activation of SREBP-1c[78]. In obese rodents, liver lipogenesis is associated with the activation of forkhead box O1 (FOXO1) signalling[287]. Recently, activation of FOXO1 has been identified to influence VLDL metabolism by increasing the expression of microsomal triglyceride transfer protein (MTTP) and the secretion of VLDL-TG[288]. It has long been known that, under fasting conditions, FOXO1 expression is increased and promotes hepatic gluconeogenesis, whereas under fed conditions, insulin inhibits the effects of FOXO1 on hepatic gluconeogenesis[289, 290]. The effect of FOXO1 on promoting VLDL production shows that FOXO1 plays a critical role in the metabolic adaptations necessary to respond to fasting and refeeding and influences metabolic flexibility. In insulin resistant obese subjects, the regulation of FOXO1 activity by insulin is impaired thereby causing both hyperglycemia and hypertriglyceridemia[291].

Another feature of the MetS is systemic low-grade inflammation. In obese individuals, adipose tissue expands resulting in the release of less anti-inflammatory and more pro-inflammatory adipokines from adipocytes[292]. As a result, the adipose tissue becomes inflamed and this is believed to play a role in the low grade systemic inflammation seen in obese subjects. Several studies have shown that pro-inflammatory cytokines can induce insulin resistance[293, 294], which may be reversed by anti-inflammatory medication[295, 296]. It seems likely that the double role of nuclear receptors in both energy/lipid metabolism and inflammation is central to the pathology associated with the MetS. Since nuclear receptors play a key role in metabolic flexibility it is interesting to

determine to what extent they also modulate our inflammatory responsiveness. In fact, while the prevalence of obesity increases in our population so does the incidence of allergy. Although there is controversy about the relation between obesity and allergy symptoms[297, 298, 299, 300], it is suggested that obesity is associated with predisposition to allergy, especially allergy for food, and that systemic inflammation plays a key role in this association[301]. It would be interesting to determine whether allergy increases the risk to develop obesity or obesity increases the risk to develop allergy.

Concluding remarks

Regulation of VLDL-TG metabolism plays a role in energy homeostasis which is disturbed in obesity and associated pathology. Nuclear receptors are important in the regulation of VLDL-TG metabolism and therefore interesting targets for the development of drugs to treat MetS. The overall focus of this thesis was on the effects of several nuclear receptors on VLDL-TG metabolism, but these nuclear receptors clearly also affect inflammation. Since the MetS is a multifactorial disease, targeting both energy homeostasis and inflammation with nuclear receptors agonists may be an excellent approach to treat obesity related diseases. Unfortunately, most studies neglect the pleiotropic effects of nuclear receptors and therefore more integrated research focussed on both lipid metabolism and inflammation is necessary.