The flow of metabolic intermediates through biochemical pathways ('metabolic fluxes') are to a certain extent controlled by 'nutrient sensors'. These sensors enable adequate adaptation to changes in nutrient availability and therefore contribute to the maintenance of energy homeostasis. Transcription factors comprise a subgroup of nutrient sensors. Chronic energy oversupply and nutritional dysbalance evoke persistent modifications in physiological processes. These adaptive responses may in the long term predispose to the development of metabolic diseases.

Ligand-activated nuclear receptors are master regulators of whole-body metabolism: they control the expression of genes encoding enzymes that constitute biochemical pathways. As such, they are considered as putative drug targets to correct metabolic disturbances such as dyslipidemia and insulin resistance. Insight into the body's adaptive physiological responses to changes in nutrient availability, and the role of transcriptional regulators herein, is needed to define optimal strategies for disease prevention and treatment.

Research described in this dissertation addresses the metabolic consequences of changes in nutrient availability. Stable isotope methodology was applied to quantify the actual metabolic fluxes in vivo and outcome was related to biochemical and gene expression analysis in relevant organs and tissues.

**NOVEL INSIGHTS INTO THE ACTION OF NUTRIENT-SENSING TRANSCRIPTION FACTORS**

Studies described in this dissertation provide new insights into the action of a specific set of transcription factors.

Oxysterol activation of LXRα promotes cellular cholesterol disposal, by inducing the expression of genes encoding transporters and enzymes that mediate cholesterol efflux, cholesterol excretion as well as cholesterol conversion into bile acids [218]. Furthermore, it has been shown that both glucose and G6P are able to bind to and activate hepatic LXR at physiological concentrations in vitro [53]. This issue has, however, been heavily debated [65–67]. In **Chapter 2** we have tested the physiological relevance of the postulated hepatic glucose sensing function of LXR in mice. We found that the induction of lipogenic genes in liver and the increase of VLDL-TG concentrations upon carbohydrate refeeding observed in wild-type mice were markedly blunted in Lxrα-/− mice. However, we did not observe any effect of either carbohydrate refeeding or Lxrα disruption on the expression of the LXR target genes Abca1 and Abcg5/8. The disruption of Lxra did furthermore not affect hepatic and peripheral insulin sensitivity, thereby confirming previous studies [60]. The blunted lipogenic response in carbohydrate-refed Lxra+/− mice was therefore most likely related to an impaired SREBP-1c action. We also noticed that the hepatic response to fasting was hampered in Lxra+/− mice. Hepatic G6P turnover was reduced and glycogen depletion was delayed. Fasting-induced steatosis was also markedly less pronounced in these animals. In contrast to the impaired lipogenic induction upon refeeding, the reduction in fasting-induced steatosis most likely results from the absence of Lxra per se, since fasted Srebp-1c−/− mice accumulated similar amounts of hepatic TG as compared to their wild-type littermates [21]. Because hepatic LXRα was found to be insensitive to dietary glucose, we hypothesize that the impaired hepatic response to fasting in Lxra+/− mice may rather be related to other metabolic changes associated with fasting, such as reduced energy availability and/or in-
creased NEFA influx and catabolism. Interestingly, LXRα has been implicated in the regulation of adipose tissue lipolysis [219]. However, the absence of LXRα may also increase RXR availability for other nuclear receptors, such as PPARα [220]. Increased PPARα action consequently promotes NEFA catabolism [13,14,102]. Thus, instead of its anticipated involvement in the regulation of hepatic metabolism in response to glucose and insulin [53,57] we have identified LXRα as a mediator of the adaptive response to fasting in the liver. This has recently been confirmed by others (Sokolovic et al., personal communication).

FXRs are activated by bile acids. Upon activation, FXR suppresses bile acid synthesis while bile acid disposal is promoted [221]. Hepatic FXR expression and transcriptional activity have also been reported to be induced by glucose [88] and the hepatic response to short-term fasting is impaired in Fxr−/− mice [44]. This has been suggested to result from an inadequate induction of gluconeogenesis. In Chapter 3, we report another feature of altered physiological responsiveness to changes in glucose availability in Fxr−/− mice. The appearance of glucose entering the blood compartment during the initial phase of (intestinal) glucose uptake was shown to be delayed in these animals. Using a combination of orally and intravenously administered isotopically labeled glucose, we showed that this delay was caused by an increased glucose flux through G6P in the enterocytes. Although speculative, this may serve to restore the enterocyte’s depleted G6P stores observed in fasted Fxr−/− mice (i.e., prior to the glucose load). Taken together, these data suggest that besides the incapability to exert feedback regulation in response to increasing bile acid concentrations, FXR inactivation is characterized by impaired sensing of a reduced glucose availability by liver and intestine. This presumably results from inadequate regulation of de novo synthesis and partitioning of G6P [44].

Activation of PPARα by fatty acids and their derivatives [12] results in an induction of genes encoding enzymes involved in their transport and catabolism [102–104]. PPARα has emerged as an important mediator of the hepatic response to fasting by ensuring energy supply through fatty acid oxidation when glucose availability is low [13,14]. In addition, PPARα protects against damage from fatty acid oxidation products by promoting anti-oxidant action and mitochondrial uncoupling [108,113,114]. In Chapter 4, we describe a novel metabolic consequence of pharmacological PPARα activation. We observed a strong induction of genes encoding enzymes involved in hepatic fatty acid synthesis and elongation in mice treated with the PPARα agonist fenofibrate. Using a novel stable isotope approach, we quantified the actual lipogenic flux and chain elongation in TG-derived fatty acids. Both de novo lipogenesis and fatty acid elongation were massively induced upon PPARα activation. Evaluation of hepatic carbohydrate fluxes and gene expression levels indicated that acetyl-CoA supply from glucose was reduced. Acetyl-CoA from fatty acid oxidation must therefore have been the major lipogenic substrate. Using specific knockout mice treated with the pharmacological PPARα agonist, we found that the induction of lipogenic genes depended on SREBP-1c but not on ChREBP. Srebp-1c expression itself was not induced upon pharmacological PPARα activation. The lipogenic induction must therefore have resulted from an increased SREBP-1c activity, as has been proposed before [119]. Interestingly, PPARα agonists fail to induce SREBP-1c activity and lipogenic gene expression in hepatoma cells [131] as well as in Pparα−/− mice [119]. These observations strongly suggest that an enhanced hepatic fatty acid influx (in response to PPARα/FGF-21-mediated fatty acid mobilization) modifies hepatic intracellular lipid status, which in turn promotes SREBP-1c action. Although PPARα is an important regulator of the adaptive response to fasting, the
physiological consequences of pharmacological PPARα activation cannot be directly extrapolated to the fasting situation, because it is generally accepted that SREBP-1c action is limited under these conditions [222,223]. Furthermore, PPARα agonists induce both mitochondrial and peroxisomal fatty acid oxidation, while during fasting, mitochondrial fatty acid oxidation predominates. Acetyl-CoA from both sources has been reported to serve as a substrate for fatty acid synthesis [134,135]. The relevance of the parallel existence of fatty acid oxidation and synthesis/elongation systems under fasting conditions remains to be established. Altogether, our data support the co-existence of hepatic β-oxidation and lipogenesis, thereby challenging the classical view that fat oxidation and synthesis are two opposing biochemical processes that occur under different metabolic conditions. We propose the PPARα/SREBP-1c-mediated induction of hepatic fatty acid synthesis and elongation as a novel physiological mechanism by which the liver is protected against fatty acids and their oxidation products.

ADAPTIVE PHYSIOLOGICAL RESPONSES TO A CHRONIC OVERLOAD OF DIETARY FAT

Glucose and fatty acids exert direct substrate competition at the cellular level [11]. This normally coincides with the availability of glucose during the postprandial and postabsorptive phases. An increase in circulating NEFA concentrations acutely inhibits glucose utilization in vivo [224–226]. This indicates that removal of circulating fatty acids and their subsequent oxidation occurs at the expense of glucose disposal.

We evaluated the effects of an increased dietary fat supply on whole-body substrate utilization (Chapter 5). Under normal conditions, carbohydrate oxidation provides the major energy supply in the postprandial phase (~85%) while the contribution of fat oxidation increases during the postabsorptive state. In mice fed a regular low-fat chow diet, this is illustrated by a switch from carbohydrate oxidation (indicated by high RER values) during the dark phase (in which the animals are active and consume most of their food) to fat oxidation (indicated by low RER values) during the light (or inactive) phase. We observed that this physiological substrate switching was abolished when mice were challenged with a hypercaloric high-fat diet (that still contained a considerable amount of glucose). Instead, these animals exhibited a persistent reliance on fat oxidation. This phenotype was even more pronounced when mice were fed a diet in which part of the saturated fat was isocalorically replaced by fish oil. Fish oil is a source of n-3 PUFA. Compared to other types of fatty acids, n-3 PUFA exert a relatively high ability to bind PPARα and –δ [12], which may explain the additional reliance on fat oxidation in fish oil-fed mice.

Consequences for glucose fluxes

The reduced glucose-to-fat oxidation ratio in both high-fat and high-fat/fish oil-fed mice was paralleled by elevated plasma insulin concentrations, suggestive for insulin resistance. We therefore evaluated the effects of these diets on whole-body glucose disposal and production in Chapter 5. Using hyperinsulinemic euglycemic clamps, we found that insulin-stimulated glucose disposal was impaired in high fat-fed mice. Insulin’s ability to suppress endogenous glucose production was also reduced in these animals. The additional decrease in the glucose-to-fat oxidation upon fish oil replacement was associated with a further deterioration of insulin-stimulated glucose disposal.
**Consequences for lipid fluxes**

Besides a sustained reliance on fat oxidation, we observed a counterintuitive induction of genes encoding enzymes involved in fatty acid synthesis and elongation in the livers of high fat-fed mice (Chapter 6), as had been reported by others [130,185]. The physiological relevance of these changes had however not been established. Fish oil, on the other hand, is known to suppress the expression of lipogenic genes by interfering with the action of the lipogenic transcription factors SREBP-1c, LXR and ChREBP [20,29,30,33,227]. Fish oil replacement indeed reduced the expression levels to values comparable to or below the chow-fed animals. We quantified the actual lipogenic flux and determined the contributions of the de novo lipogenesis and chain elongation of pre-existing palmitate.

High-fat feeding resulted in a more than twofold increase in oleate synthesis. De novo synthesized palmitate, however, only minimally contributed to this increase. Instead, elongation of pre-existing palmitate mainly accounted for the induction of lipogenesis upon high-fat feeding. High-fat feeding furthermore induced cholesterol synthesis, presumably to ensure cholesterol supply for fatty acid esterification. These adaptations resulted in the accumulation both of TGs and CEs, which was progressive since there was no compensatory increase in hepatic VLDL-TG secretion. Fish oil replacement of the saturated fat suppressed de novo lipogenic flux, almost completely abolished fatty acid elongation and normalized cholesterol synthesis. As such, it prevented the high fat diet-induced accumulation of TGs and CEs. It furthermore resulted in an inhibition of VLDL-TG secretion, which contributed to the lowering of TG concentrations in the plasma. Although the hepatic lipogenic gene expression profiles correlated well to the amount of hepatic TG and the total lipogenic fluxes, hepatic lipid accumulation in high-fat fed mice rather resulted from an increased fatty acid elongation than from an induction of de novo lipogenesis.

**ENERGY OVERSUPPLY, NUTRITIONAL DYSBALANCE AND THEIR PATHOPHYSIOLOGICAL CONSEQUENCES**

*Obesity and lipid overflow*

The body does not reduce its energy intake in response to fat oversupply; intestinal fatty acid absorption is actually increased in response to high-fat feeding in mice [228]. This response may serve to limit exposure of enterocytes to NEFAs. Obesity results from a chronic imbalance between energy intake and energy expenditure, and is associated with an enhanced release of NEFA into the circulation. The influx of fatty acids to organs is consequently increased. Such 'lipid overflow' from adipose to non-adipose tissues is generally considered as a crucial event in the development of the metabolic diseases.

Recent studies point to the storage capacity of the adipose tissue, and not the absolute amount of fat per se, as an important determinant in the development of metabolic complications [229–231]. Lipid overflow may therefore not to be directly related to fat mass, but rather reflects an inability to store fat in adipocytes. This may explain why in some cases, the severity of complications in extreme obese individuals is less than in those with a mild degree of obesity. Expansion of the fat storage capacity in adipose tissue has been shown to improve insulin sensitivity [231,232], despite a concomitant increase in adiposity. On the other hand, an impaired ability to store fat in the adipose tissue results in global metabolic failure [230,233,234].
Metabolic inflexibility and insulin resistance

Metabolic flexibility is defined as the ability of a system to adjust fuel utilization to fuel availability. The switch in fuel utilization will depend on the type and amount of nutrients available at the cellular level [235]. Increased circulating NEFA levels will increase the amount of fatty acid available for oxidation and subsequently impair glucose oxidation.

Physiological switching between carbohydrate and fat during active (fed) and inactive (fasting) phases is abolished upon high-fat feeding in mice (Chapter 5). Such an adaptive shift in substrate utilization is already observed upon acute exposure to high-fat diets (van den Berg et al., personal communication), and thus occurs independently of obesity associated with long-term high-fat feeding. It illustrates the dominance of fat oxidation over glucose oxidation, which may again reflect an attempt of the body to limit lipotoxic damage. This response, however, induces metabolic inflexibility to glucose. Because the high-fats also contain carbohydrates, blood glucose concentrations will rise. This is illustrated by the hyperglycemia observed in high fat-fed mice. The elevated glucose concentrations subsequently trigger insulin release, resulting in hyperinsulinemia. In addition, the body’s responsiveness to insulin (i.e., insulin sensitivity) decreases, indicated by the impaired ability of chronic hyperinsulinemia to restore glucose disposal rates in high fat-fed mice. This is presumably related to diet-induced changes in membrane fatty acid composition affecting IR/IRS-P13K action [236–238]. IRS-dependent insulin signalling is furthermore reduced by an increase in intracellular lipid species such as long-chain fatty acid-CoA, diacylglycerol and ceramide [239]. We indeed observed an impairment in the insulin-mediated increase in IRS-associated P13K activity in adipose tissue and liver upon high-fat feeding (Chapter 5). The translocation of GLUT4 to the plasma membrane is consequently impaired, and glucose uptake is reduced. Interestingly, a single high-glucose low-fat meal has been reported to restore insulin-stimulated glucose disposal in skeletal muscles of rats maintained on a high-fat diet during three weeks [236,240].

Human studies support the hypothesis of metabolic inflexibility to glucose as an initial event in the pathogenesis of insulin resistance and type 2 diabetes. The ability to switch from fat oxidation to carbohydrate oxidation after a meal is impaired in the prediabetic (i.e., glucose intolerant) state [172] and metabolic inflexibility is predominantly related to defective glucose disposal in type 2 diabetic subjects [174]. Furthermore, it is partially reversed by weight loss [172,174]. Besides a reduction of caloric intake, restoration of nutrient balance may therefore be an effective approach to improve glucose tolerance and insulin sensitivity. This implies that fat intake should be restricted to ~30% of total energy, while glucose should not be provided as simple sugars, but as complex carbohydrates.

Hepatic steatosis

Dietary fat oversupply and obesity increase the flux of NEFA towards the liver. This promotes hepatic lipid accumulation, as re-esterification of circulating NEFA comprises a major contribution to the hepatic TG pool [241,242]. Work described in Chapter 6 provides new insights into the adaptive physiological responses to an increased hepatic NEFA influx. We have shown that in high fat-fed mice, the liver elongates pre-existing palmitate, which is subsequently desaturated to facilitate its storage as TG, while VLDL-TG output remains unaltered. Although oleate synthesized via this pathway only comprises a minor part of the total pool, fatty acid elongation may significantly contribute to steatosis progression in the long term. The relevance of this finding is illustrated by the observation that a high-fat diet increases liver fat content in human subjects without affecting body fat [182]. High-fat

Chapter 7 113
feeding furthermore promotes cholesterol synthesis, which results in the accumulation of hepatic CEs.

**Figure 1.** Overview of the adaptive physiological events in the development of lipid-induced insulin resistance.

Reduction of dietary fat intake and weight loss limit the hepatic NEFA influx and consequently arrest hepatic lipid accumulation [182,243]. Consumption of fish oil promotes fat oxidation, thereby reducing the amount of fatty acids available for hepatic re-esterification. Specific types of fatty acids furthermore affect LPL-mediated chylomicron clearance [211], thereby reducing fatty acid spill over from chylomicron-derived TGs to the blood compartment [244]. The intake of n-3 PUFA may thus sequester dietary fatty acids in adipose stores [209,210], thereby reducing lipid storage in the liver. Finally, expansion of the adipocyte’s fat storage capacity upon TZD treatment improves liver function in NAFLD patients [245], presumably by preventing lipid overflow. Long-term continuation of TZD therapy is required to maintain improvements in morbidity [246]. This is however accompanied by undesired weight gain.

**HEPATIC LIPID SYNTHESIS AS AN ADAPTIVE PHYSIOLOGICAL RESPONSE TO AN INCREASED FATTY ACID INFLUX. A ROLE FOR TRANSCRIPTION FACTORS?**

Hepatic storage of NEFA and acetyl-CoA from β-oxidation as relatively harmless TGs may represent a physiological mechanism to prevent lipotoxic damage (Chapter 4 and 6; [230,247]). SCD1 action appears to play a crucial role in this protective action, because it is required for the partitioning of excess NEFA into MUFA, which in turn can be safely stored as TGs [100]. Hepatic Scd1 expression is induced upon high-fat feeding in mice (Chapter 6 and [100,130]) and in human fatty liver [248]. Inhibition of SCD1 action increases susceptibility to saturated fat-induced apoptosis. Hepatocellular
apoptosis, liver injury, and fibrosis are markedly increased in Scd1^{-/-} mice challenged with a methionine-choline-deficient, while steatosis is decreased in these animals [100]. This supposed protective SCD1 action is not restricted to the liver [249].

The induction of hepatic SCD1 action in response to oversupply of dietary fat may be secondary to increased transcription factor action. PPARs are likely candidates to mediate these adaptations, because they are activated by fatty acids. Scd1 has actually been reported as a direct target gene of PPARs [120,250]. Furthermore, Ppary2 is ectopically induced in liver in response to overfeeding [251,252]. PPARy2 action has been reported to prevent lipotoxicity by facilitating the deposition as fatty acids as TG: ablation of Ppary2 in ob/ob mice results in an increased hepatic ceramide content, in parallel to a reduction in TGs [230]. The studies described in Chapter 3 and 6 point to a regulatory role for SREBP-1c, as has been suggested by others [185]. Evidence for an inadequate response to high-fat feeding in the absence of SREBP-1c is however not available. Experiments involving Srebp-1c^{-/-} mice will provide more insight into the potential role of this transcription factor in the development of high-fat diet induced hepatic steatosis.

Interestingly, the lipogenic transcriptional regulators PPARγ2, LXR and SREBP-1c [46–48] have all been reported to be over-expressed in human fatty liver. Hepatic upregulation of transcription factor expression may therefore reflect an attempt to protect the liver against fatty acid oversupply. It should however be noted that rather harmless TG accumulation may eventually predispose to development of liver disease, because it renders the liver more susceptible to 'second hits'[181].

![Figure 2](image-url) Proposed mechanism by which an increased flux of fatty acids towards the liver promotes hepatic lipogenesis and TG storage via the action of nutrient-sensing transcription factors.
CONCLUSIONS AND IMPLICATIONS

The metabolic syndrome comprises a number of disturbances in energy and nutrient metabolism including obesity, insulin resistance and dyslipidemia. Progression of these disturbances will ultimately lead to type 2 diabetes and cardiovascular disease. The underlying pathophysiological mechanisms are multifactorial. Prevention and/or treatment of this multimorbidity requires a global approach, including the simultaneous modulation of multiple metabolic processes.

Transcription factors adjust the expression of metabolic enzymes in response to changes in nutrient availability. A change in the availability of metabolic enzymes may consequently affect the flux through a biochemical pathway. The possibility to modulate metabolic fluxes via the action of ligand-activated nuclear receptors has sparked the interest to design drugs that act on these regulators. This requires insight into the physiological consequences of transcription factor action.

When considering a nuclear receptor as a potential drug target, one should be aware that metabolic remodelling may provoke undesired side-effects. An alteration of a specific biochemical reaction will affect the flux through the entire pathway. In addition, the effect of a specific enzyme on the global flux may furthermore vary under different metabolic conditions [52]. Changes in the expression and/or abundance of metabolic enzymes may thus not always truly reflect the actual metabolic fluxes, and the physiological consequences of transcription factor action may therefore be different than what is predicted from gene expression patterns. One should also be careful to draw general conclusions on transcription factor action based on experimental evidence obtained under specific conditions. In vivo evaluation of drug action is furthermore required to obtain a complete physiological picture. This is of particular importance for multidrug approaches required to prevent and/or treat the multimorbidities that comprise the metabolic syndrome.

These issues underline the need to apply fluxomics in vivo, thereby establishing the physiological relevance of a ‘static snapshot’ obtained from genomic, proteomic and metabolomic approaches [35,253]. We have combined genomics, metabolics and fluxomics to add to the current understanding on the regulation of metabolic fluxes by specific transcription factors. The results will contribute to the development of new drugs to prevent and/or treat metabolic disturbances such as dyslipidemia and insulin resistance. However, in most cases, transcription factors are expressed in multiple organs. Global targeting of these regulators may consequently induce unwanted physiological responses. Dissection of the tissue-specific actions of transcription factors is of particular importance to identify undesirable side-effects of drug treatment. Therefore, possibilities for tissue-specific targeting have to be explored and optimized. In addition, the application of metabolic pathway analysis is of particular importance to test the usefulness of potential therapeutic strategies, and to discover novel drug targets [254,255].