Summary and Synthesis

Concluding remarks and Future perspectives
SUMMARY AND SYNTHESIS

The World Health Organization (WHO) has estimated that to date the worldwide number of overweight adults has reached a staggering 1 billion of which at least 300 million are clinically obese. Strikingly, there is a significant increase in the proportion of obese children and adolescents. Obesity is a major contributor to the global burden of the metabolic syndrome and chronic diseases like type 2 diabetes and cardiovascular disease (CVD). Although the obesity pandemic is most likely driven by the worldwide “food transition”, i.e., excess intake of diets with high contents of fat and sugar in conjunction with a sedentary lifestyle, the etiology of obesity-related diseases is complex and has so far remained to certain extent elusive. Metabolism involves an intricate crosstalk between various metabolic pathways such as glucose, fatty acid and bile salt metabolism. Interruption of one pathway often leads to (irreversible) changes in another pathway. Insight in regulation of interrelated metabolic pathways in (patho)physiology can be obtained from mouse models for diabetes, for deficient mitochondrial fatty acid oxidation (mFAO) and application of drugs and diets. Studies described in this thesis provide insight in crosstalk between glucose, fatty acid and bile salt metabolism that is relevant for our understanding of the etiology and some of the consequences of metabolic diseases.

In Chapter 2 and 3, the effects of bile salt sequestration on lipid and glucose metabolism in lean and diabetic db/db mice have been addressed. We show that despite massive fecal bile salt loss, bile salt pool size can be maintained due to complete compensation by de novo synthesis of bile salts. Nevertheless, sequestrant-treated mice had increased liver triglyceride contents which was mainly periportally confined. Sequestrant-treatment improved glucose levels in db/db mice by increasing metabolic clearance rates of glucose. The glucose lowering effects are, therefore, not directly related to changes in hepatic carbohydrate metabolism.

In a next series of experiments, described in Chapter 4, we have assessed the effects of dextrose feeding on bile salt metabolism. Dextrose feeding led to a strong reduction in bile salt synthesis and pool size. We suggest that this effect might be due to enhanced Fgf15 expression, likely mediated by diet-induced effects on FXR activity.

Chapter 5 and 6 describe the consequences of disturbed fatty acid oxidation in Mcad+/− and Lcad+/− mice, respectively, on glucose metabolism. Despite hypoglycemia upon fasting but particularly in sepsis, de novo synthesis of G6P (gluconeogenesis) was unaffected in Mcad+/− mice. Metabolic clearance of glucose accounted for the hypoglycemia observed. Also in Lcad+/− mice, we did not find affected gluconeogenesis upon fasting. Increased metabolic clearance rates of glucose in Lcad+/− mice compared to their wild-type littermates accounted for the hypoglycemia upon fasting. These results demonstrate that peripheral rather than hepatic consequences of mFAO underlie hypoglycemia with disorders of mFAO.
The concept of metabolic zonation of hepatic metabolism has been assessed in Chapter 7. We specifically focused on zonation of PEPCK mRNA and protein expression in livers of control and Alloxan-induced type 1 diabetic mice using several approaches, *i.e.*, laser dissection microscopy (LDM), digitonin-collagenase perfusion and immunohistochemical staining.

**BILE SALTS: MORE THAN SOAP!**

**Bile salt metabolism in diabetes**

Over the past two decades, the view on bile salts as functioning mainly as “intestinal soap” has changed in to the view that bile salts act as important molecular modulators of metabolism. Bile salts regulate the expression of several genes involved in numerous aspects of their own enterohepatic circulation. Additionally, bile salts have been found to integrate energy, glucose and fatty acid metabolism. Bile salt metabolism is significantly altered in diabetes. So far, it is unknown whether these alterations also contribute to the metabolic derangements seen with the disease. Additionally, the mechanisms underlying affected bile salt metabolism in diabetes are insufficiently known. Studies in animal models for diabetes revealed significant insight in regulatory mechanisms. In spontaneous and streptozotocin-induced type 1 diabetic rats, bile salt pool sizes are increased. Interestingly, insulin restores plasma bile salt concentrations in diabetic rats and in liver-specific insulin receptor knockout (LIRKO) mice, two widely used mouse models for type 2 diabetes. In leptin-deficient *ob/ob* mice, another mouse model for type 2 diabetes, leptin administration improved insulin sensitivity and also decreased bile salt pool size. Based on these and older observations, disturbed hepatic insulin signaling has been suggested to directly contribute to changes in bile salt synthesis. Mechanistic insight in insulin-mediated control of bile salt metabolism came from observations that the rate-limiting enzyme in bile salt synthesis, *Cyp7a1*, is a direct target of the insulin-regulated transcription factor FOXO1. Insulin phosphorylates FOXO1 which causes the protein to retract from the nucleus, thereby reducing its transcriptional control on nuclear targets like *Cyp7a1*. Adenovirus-mediated overexpression of FOXO1 and loss-of-function using *Foxo1* siRNA resulted in inhibition and stimulation, respectively, of *Cyp7a1* gene expression in human hepatocytes. In the same study, FOXO1 was shown to directly interact with HNF4a to reduce PGC-1α occupancy in *Cyp7a1* chromatin. PGC-1α has pleiotropic effects on the *Cyp7a1* promotor. In type 1 diabetes, *i.e.*, in the absence of insulin, and in type 2 diabetes, with impaired insulin signaling, FOXO1 is not phosphorylated and remains in the nucleus where it activates *Cyp7a1*. Interestingly, upon fasting, when insulin levels are low, hepatic *Cyp7a1* expression is increased. Remarkably, intestinal *Fgf15* expression is also increased under fasting conditions. *Fgf15* exert its action via FGFR4 expressed in the liver. It has been...
shown that hepatic Fgfr4 mRNA and protein are increased upon insulin stimulation and decreased in STZ-induced diabetic mice which lack insulin \(^{13}\). Thus, when insulin concentrations are low, FGFR4 levels are low which prevents Fgf15 to exert its inhibitory action on Cyp7a1 expression. FGFR4-signaling, like insulin signaling, activates the PI3-kinase signaling pathway. This partly explains how FGF19 administration seems to impact on hepatic insulin signaling \(^{14,15}\). In contrast to fasting, Fgf15 is not detectable in ilea of Alloxan-induced diabetic mice (Brufau G, unpublished data) which lack insulin. These data suggest that insulin plays an important but yet unidentified role in intestinal Fgf15 metabolism. Clearly, the molecular mechanisms regulating bile salt metabolism in diabetics but also in healthy people are complex. Although several redundant pathways have been hypothesized \(^{16}\), many remain to be resolved.

**Bile salt and lipid metabolism**

Bile salts are synthesized from cholesterol in hepatocytes. This conversion quantitatively represents the major route for cholesterol removal from the body. For many years it has been known that increasing this conversion, e.g., by bile salt sequestration, significantly improves cholesterol levels in hypercholesterolemic patients \(^1\). Yet, use of bile salt sequestrants has also been associated with elevated plasma triglyceride levels in humans. The mechanism of this hypertriglyceridemic effect has remained unresolved so far. Limited functional animal studies in which the effects of bile salt sequestrants on lipid metabolism have been studied are available \(^{17,18}\). Additionally, these studies have been carried out in high-fat diet-fed mice in which part of the beneficial effects of sequestrant feeding are likely attributable to malabsorption of dietary fat. The objective of Chapter 2 was to assess the effects of bile salt sequestration on bile salt and fatty acid metabolism in chow-fed lean and obese, diabetic db/db mice. Importantly, sequestrant-treatment did not affect bile salt pool size. Nevertheless, lipogenesis and hepatic triglyceride content were markedly increased as demonstrated with a novel stable isotope techniques. Since lipogenesis is a pericentral process within the liver, it was remarkable to find triglycerides mainly in periportal areas. Although we show that the lipogenic response is, at least at the gene expression level, dependent on LXR and FXR signaling, the exact molecular mechanism remains elusive.

We hypothesized that reduced bile salt concentrations in periportal areas of sequestrant-treated mice compared to control mice could in part underlie the portal distribution of triglycerides. Possibly, lipogenic pathways which are reduced by bile salt-signaling under normal conditions are activated by less effective bile salt signaling upon bile salt sequestration. Bile salts are taken up efficiently by periportal hepatocytes thereby creating a decreasing concentration gradient along the sinusoidal space. Consequently, periportal hepatocytes are exposed to a six-fold higher concentration of bile salts as compared with pericentral cells \(^{19}\). Sequestrant-
treatment reduced periportal bile salt concentrations by ~30% which likely affects bile salt-mediated signaling in periportal hepatocytes (Figure 1).

Distribution of the bile salt regulatory gene \textit{Cyp7a1} has been shown to respond to changes in periportal bile salt concentrations. \textit{Cyp7a1} expression is normally limited to centrally located hepatocytes but translocates to a larger area of the liver lobulus with more involvement of periportally located cells upon sequestrant treatment. Contrary, expression of \textit{Hmgr}, the rate-limiting enzyme in cholesterol synthesis that is massively upregulated upon sequestrant treatment, remains periportally localized. Although the response to changes in bile salt concentrations is diverse, it might explain in part the metabolic changes upon sequestrant treatment. The contribution of \textit{de novo} synthesized bile salts to bile salt pool size is massively increased in treated compared to untreated mice. The capacity of newly synthesized bile salts to immediately act on signaling pathways is unknown.

\textit{Fxr} mRNA is homogenously distributed over the liver acinus. The actions of this nuclear receptor are therefore posttranscriptionally regulated by concentrations of its ligands, \textit{i.e.}, bile salts. The role of FXR in regulation of lipid metabolism has been discussed in Chapter 2 and has been extensively reviewed by us (Hageman/Herrema review, ahead of publishing). Reduced FXR activation in periportal areas of sequestrant-treated mice might reduce the suggested inhibitory role of FXR-SHP on LXR-induced activation of the lipogenic transcription factor SREBP1c. Cholesterol synthesis, a periportal process, is massively increased upon sequestrant treatments and might affect lipid metabolism specifically in this area. It could therefore be hypothesized that oxysterols, derivatives of cholesterol, activate LXR, one of the key regulators of SREBP1c. Lxr appeared to be a prerequisite for the increased

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\textbf{Figure 1.} Bile salt sequestration reduces bile salt-mediated signaling in periportal hepatocytes. Periportal bile salt concentrations are reduced by ~30% upon sequestrant treatment which possibly affects bile salt-mediated signaling pathways in this zone. Additionally, there is a shift from reabsorption of bile salts in control mice to \textit{de novo} synthesis upon sequestrant treatment.
lipogenic gene expression upon sequestrant-treatment (Chapter 2). To date, there is no information available on zonation of LXR. Increased cholesterol synthesis might lead to increased synthesis of oxysterols and hence, activation of LXR in periportal areas. Another hypothesis is that the relative deprivation of hepatic microsomal cholesterol content upon bile salt sequestration activates the lipogenic pathway. Although hepatic cholesterol content of sequestrant-treated mice was unchanged, the turnover of cholesterol was massively induced as indicated by increased fractional de novo cholesterol synthesis and cholesterol conversion into bile salts. The cholesterol molecules available in the endoplasmic reticulum (ER) membrane for inhibition of proteolytic cleavage of sterol regulatory element-2 (SREBP2), a key-regulator of cholesterol homeostasis, are consequently reduced thereby mimicking a condition of deprived cholesterol content. Low sterol levels induce proteolytic cleavage of SREBP2 and enhance translocation into the nucleus where it activates transcription of its target genes. Via this mechanism, bile sequestration increases the expression levels of LDLR and HMGR. A similar mechanism could be proposed for SREBP1c. SREBP1c is generated as inactive precursor in ER membranes where it is captured by SREBP cleavage-activating protein (SCAP). Upon cleavage from the ER, the SCAP-SREBP1c complex migrate to the Golgi. SREBP1c is activated in this organelle by proteolytic processing. The massive turnover of cholesterol and subsequent depletion of cholesterol from the ER, might induce SCAP-SREBP1c migration thereby increasing the levels of active SREBP1c (Figure 2). Detailed studies addressing the consequences of induction of cholesterol turnover on ER membrane homeostasis.

![SCAP-SREBP1c complex migration](image)

**Figure 2.** Relative depletion of cholesterol from the ER membrane upon sequestrant-treatment might induce migration of the SCAP-SREBP1c complex.
and, hence, on SREBP1c activation might provide valuable insight in the lipogenic response to bile salt sequestration.

**Bile salts as regulators of glucose metabolism**

Bile salt sequestration has beneficial effects on blood glucose and HbA1c levels in type 2 diabetes. For these effects, bile salt sequestrants were the first FDA-approved drugs to treat both cholesterol and glucose metabolism in diabetics. Nevertheless, the underlying mechanism of improved glucose homeostasis is yet to be determined. In Chapter 3 we have questioned whether the improved blood glucose profile upon sequestrant-treatment was due to direct action of affected bile salt signaling on hepatic glucose metabolism. For this purpose, glucose metabolism was studied in sequestrant-treated lean and diabetic db/db mice by application of stable isotope techniques. Although hepatic glucose fluxes were clearly altered upon sequestrant-treatment, the decreased blood glucose levels were mainly attributable to increased metabolic clearance rates in sequestrant-treated db/db mice. An improved HOMA-index in treated diabetic mice was suggestive of improved insulin sensitivity. It has been suggested that muscle insulin resistance arises from impaired mitochondrial uptake and oxidation of fatty acids. Interestingly, we found reduced long-chain acylcarnitine content in skeletal muscles of db/db mice upon sequestrant treatment. Acylcarnitines are acyl esters of carnitine and the transport form of fatty acids across the mitochondrial membrane. Increased long-chain acylcarnitines can reflect an inability of mFAO to run efficiently leading to accumulation of intermediate acyl-CoA's in mitochondria. These intermediates are potentially toxic and are exported out of the mitochondria upon acyl transfer to carnitine. In inborn errors of mFAO, acylcarnitines are typically found in high concentrations in tissues, plasma and bile. The subsequent high levels of long-chain acylcarnitines could be preferentially converted into diacylglycerides (DAG) and ceramide which have been shown to interfere with insulin signaling. Increased levels of these intermediates, however, do not increase insulin resistance per se. Instead, mitochondrial overload and excessive instead of reduced mitochondrial fatty acid oxidation might play an important role in obesity-associated diabetes.

Plasma NEFA's were significantly reduced in sequestrant-treated db/db mice compared to their untreated counterparts. High plasma fatty acid concentrations can inhibit glucose utilization and uptake. Conversely, high plasma glucose levels can reduce fatty acid oxidation and favor fatty acid esterification. Reduction in levels of both glucose and NEFA's might reflect improved metabolic flexibility although it remains to be determined which parameter is first to change. Then, how does bile salt signaling impact on peripheral glucose metabolism? The main glucose-consuming tissues are muscle and adipose tissue. A direct effect of bile salt signaling via the bile salt receptor FXR in muscle is unlikely since this nuclear receptor is not expressed in this tissue. First evidence for a role of FXR in adipose tissue came from obser-
vations that Fxr−/− mice have reduced fat cell size and reduced peripheral insulin resistance, as determined by hyperinsulinemic-euglycemic clamps. Additionally, activation of FXR promoted adipocyte differentiation and function in vivo and in vitro. Since plasma bile salt levels were lower and results from Chapter 3 were suggestive of increased insulin sensitivity upon sequestrant treatment, it is unlikely that effects on glucose clearance are directly mediated by bile salt-mediated regulation of FXR in adipose tissue.

An FXR-independent role for bile salts in regulation of metabolism has been suggested to be mediated by modulation of local thyroid hormone production in brown adipose tissue (rodents) and muscle (humans) via the bile salt receptor TGR5. Tgr5 is expressed in a variety of tissues including muscle, central nervous system, BAT and gallbladder. Activation of TGR5 by bile salts induces the conversion of inactive into active thyroid hormone molecules via type 1 and type 2 iodothyronine deiodinase (D1 and D2). Thyroid hormones are produced by the thyroid gland and act to increase metabolic rate. However, beneficial effects of TGR5 signaling occur upon activation by bile salts, particularly hydrophobic species: since plasma bile salt were lower in sequestrant-treated animals, a role for peripheral TGR5 is unlikely. Biliary bile salt secretion was not affected upon sequestrant treatment, however, we found slight differences in biliary bile salt composition. Bile salts have been suggested to regulate metabolic rate by inducing intestinal secretion of the incretin glucagon-like-peptide 1 (GLP-1) in a Tgr5-dependent manner, as deduced from studies in enteroendocrine cells in vitro. Additionally, it has been shown that in vivo stimulation of TGR5 has beneficial effects on glucose homeostasis and increased energy expenditure via, amongst others, increased intestinal incretin secretion in mice. Since incretins have been proven effective in treating type 2 diabetes, the effects of bile salt sequestrants on intestinal incretin secretion might be worthwhile to consider.

**Glucose as regulator of bile salt synthesis**

Bile salts are important endogenous activators of FXR. Nevertheless, other factors are involved in regulation of this nuclear receptor. Glucose has been hypothesized as an important regulator of bile salt metabolism in diabetes since intermediates of the pentose phosphate pathway have been shown to increase Fxr expression levels in primary rat hepatocytes. Although we did not find differences in hepatic Fxr gene expression levels in db/db mice compared to lean mice (Chapter 2), increased and decreased Fxr expression levels were previously observed in type 2 diabetic mice and streptozotocin-induced type 1 diabetic rats, respectively. In line with the hypothesis that glucose acts on Fxr expression levels, thereby modulating bile salt metabolism, it was shown that simple sugars, impact on several aspects of bile salt metabolism in both humans and animal models. In Chapter 4, we have assessed the effects of simple carbohydrate (dextrose) feeding on bile salt metabolism.
in mice. Dextrose feeding reduced bile salt synthesis and pool size. Remarkably, despite low biliary bile salt output, expression levels of FXR-target genes Shp and Fgf15 were massively increased in ilea of dextrose-fed mice. The observed effect was, to a great extent, mediated by intestinal FXR since we found less pronounced effects of dextrose feeding in intestine-specific Fxr knockout mice (iFxr<sup>−/−</sup>). These observations were counterintuitive since low intestinal bile salt levels, expected on the basis of low biliary bile salt secretion, would result in reduced activation of FXR and, hence, in decreased expression of target genes like Shp and Fgf15. Therefore, other metabolites or processes act on FXR activity or modulate the affinity of FXR for bile salts or co-factors.

The FXR-target gene Fgf15 is mainly expressed in the distal ileum of humans and rodents. Assuming that glucose activates the FXR-FGF15 signaling pathway, the question that needs to be answered is how this would work at the intestinal level since simple carbohydrates are very rapidly taken up in the proximal ileum. One interesting hypothesis is that upon high simple sugar intake, the proximal intestine becomes relatively ‘insensitive’ to glucose. High simple carbohydrate intake leads to rapid insertion of the glucose transporter type 2 (GLUT2) into the apical side of proximal intestinal cells. Additionally, insulin secretion from the pancreas increases which, in an insulin-sensitive system, causes retraction of GLUT2 from the membrane to prevent bulk uptake of glucose. Via this mechanism it would be possible that glucose is taken up more distally in the intestine. This mechanism, which is thought to protect from acute high intracellular and systemic glucose levels, has been hypothesized to be defective in diabetes. This results in a constant and rapid inflow of glucose from the intestine which adds to the high postprandial glucose levels observed in these patients. The enigmatic high-affinity glucose transporter GLUT7 has been suggested to be responsible for glucose uptake in the terminal ileum. Since we neither measured increased glucose concentrations in distal intestinal contents nor found differences in Glut7 expression levels in terminal ilea of dextrose-fed mice compared to chow-fed mice (data not shown in this thesis), it is unlikely that regulation of glucose on FXR activity acts via the luminal site of the intestine. In favor of rejecting this hypothesis, hepatic FXR targets were also increased which is suggestive of a more general form of FXR activation.

FXR transactivation capacity is regulated at many (posttranslational) levels, amongst others via acetylation and phosphorylation. In Chapter 4, we questioned whether FXR could be posttranslationally regulated by glucose-hexosamine-derived O-linked β-N-acetylglucosamine modification (GlcNAcylation), a process similar to phosphorylation. GlcNAcylation has previously been shown to be an important regulatory mechanism for other nutrient-responsive transcription factors like LXR and FOXO1. GlcNAcylation is dependent on the concentration of UDP-GlcNAc which is produced by the hexosamine biosynthetic pathway (HBP). HBP itself depends on the amount of glucose entering a cell. GlcNAcylation is
regulated by GlcNAc transferase (OGT) and GlcNAcE, two antagonistic enzymes, which respectively add and remove a GlcNAc moiety (from UDP-GlcNAc) at serine and/or threonine sites on proteins. For further background reading, see 63. We are currently exploring the possible role of this posttranslational process in regulation of FXR. Although we currently focus on glucose as important mediator of the effects of dextrose feeding on bile salt metabolism, it is likely that other factors e.g., changes in bacterial flora due to changes in diet composition, add significantly to differences in bile salt metabolism in the studied groups.

COMMUNICATION: A VITAL PROPERTY OF LIFE

Fibroblast growth factor family in metabolic control

Members of the fibroblast growth factor (FGF) family have been implicated in regulation of a variety of metabolic processes. FGF19 and FGF21 have low heparin binding compared to other family members 64. Therefore, these FGFs are not retained in the extracellular matrix and act as endocrine hormones. FGF15, the mouse ortholog of human FGF19, is secreted from the intestine and binds to liver FGF receptor 4 (FGFR4) to inhibit bile salt synthesis via ERK-, p38-, and JNK-signaling pathways 65,66. Besides a regulatory role in bile salt metabolism, FGF19/15 has been suggested to impact on glucose and energy metabolism. FGF19 treatment of high fat diet-fed mice and transgenic overexpression of FGF19 in livers of obese, diabetic mice increased energy expenditure and decreased adipose tissue stores 67. Additionally, FGF19 has been shown to reduce insulin-stimulated fatty acid synthesis in vitro 68. The role of FGF19 in energy metabolism appears to depend on interaction with the FGFR cofactor β-klotho. Treatment of ob/ob mice with a mutant FGF19 protein lacking the β-klotho interaction domain suppressed expression of Cyp7a1 but was unable to improve glucose levels and insulin sensitivity 69. Interestingly, Fgf15 could not be detected in terminal ilea of sequestrant-treated mice whereas bile salt reabsorption was still 70% of control values (Chapter 2). Low or even absent levels of Fgf15 are, at least in part, responsible for the derepressed bile salt synthesis upon bile salt sequestration. They are, however, less likely to cause the observed metabolic consequences of bile salt sequestration since beneficial effects in the studies described were observed upon administration of the FGF19. Importantly, it is not possible to measure plasma FGF15 protein levels to date. Effects ascribed to FGF15 have been based on measurements of gene expression levels: care should be taken to speculate about FGF15-mediated effects on metabolism without adequate quantification of protein levels. FGF21, another member of the FGF family, plays an important role in regulation of energy metabolism during fasting 70. FGF21 is expressed in liver, WAT and pancreas 71,72 and has emerged as potential therapeutic target because of its antidiabetic properties. Administration of FGF21 increased metabolic rate 15 and
corrected hyperglycemia and hypertriglyceridemia in mice \(^73\) and monkeys \(^74\). Interestingly, hepatic FGF21 expression levels were increased in sequestrant-treated mice (Chapter 3). Importantly, FGF21 exerts its action via FGF receptors (kinases) complexed with β-klotho which is expressed in adipose tissue but not in muscle \(^75\). Possibly, increased glucose uptake into adipose tissue, as shown in vitro \(^76\), could lead to decreased plasma glucose levels which then improves fatty acid oxidation in muscle and, hence, decreases fatty acid content in muscle. Interestingly, FGF21 levels are increased in obese humans and mice \(^77\). Still, treatment of diabetic mice with FGF21 improves blood glucose levels \(^76\) and acutely reduces adipose tissue lipolysis \(^78\). Induction of hepatic FGF21 expression during fasting is PPARα-dependent \(^79,80\). Recently, it was shown that besides a PPARα-dependent induction of FGF21 by FFA, FGF21 is also increased by hyperinsulinemia in humans \(^81\). Thus, elevated FFA levels and hyperinsulinemia likely cause increased FGF21 levels in diabetes. The mechanisms underlying induction of FGF21 upon bile salt sequestration are yet to be determined.

**Crosstalk between fatty acid and glucose metabolism**

Coordinated crosstalk between metabolic pathways in the liver is of great importance to meet the energy demands of the body. A clear example of the importance of this crosstalk is provided by inborn errors of mFAO. Hepatic mFAO is considered to be of crucial importance for maintenance of energy homeostasis, especially during conditions of high metabolic demand such as prolonged fasting or infection. The first step of mFAO is dehydrogenation of acyl CoAs by acyl CoA dehydrogenases (ACADs). There are four dehydrogenases which act on acyl CoAs of different chain lengths, e.g., very-long-, long-, medium- and short-chain acyl CoA dehydrogenases, VLCAD, LCAD, MCAD and SCAD, respectively. The most common errors in mFAO are caused by inborn errors in these ACADs \(^82\). Patients with mFAO defects can present with severe hypoglycemia upon metabolic stress. So far, the exact pathogenetic mechanisms leading to hypoglycemia in mFAO defects have only partially been elucidated. The dogma that inhibition of β-oxidation leads to impaired gluconeogenesis was first based on observations that fatty acid oxidation increased gluconeogenesis \(^83\) and *vice versa*: inhibition of fatty acid oxidation abolished upregulation of gluconeogenesis \(^84\). We have assessed hypoglycemia upon acute infection and fasting, respectively, in two mouse models of inherited fatty acid oxidation defects, *i.e.*, *Mead*\(^{-/-}\) and *Lcad*\(^{-/-}\) mice (Chapter 5 and 6). *Mead*\(^{-/-}\) and *Lcad*\(^{-/-}\) mice both showed an unaffected *de novo* synthesis of G6P (gluconeogenesis) upon fasting. Also in the *Ppara*\(^{-/-}\) mouse, another mouse model with impaired mFAO, *de novo* synthesis of G6P has been shown to be unaffected \(^86\). Metabolic effects of LCAD deficiency appeared to be more drastic compared to those of MCAD deficiency, *e.g.*, fasting had stronger hypoglycemic effects in the *Lcad*\(^{-/-}\) mice. Additionally, hepatic glycogen stores were completely drained upon fasting in *Lcad*\(^{-/-}\) mice whereas *Mead*\(^{-/-}\) mice even showed increased glycogen contents compared to their fasted wild-type coun-
terparts. The striking difference in PDK4 expression upon fasting between both models supports the more drastic effect of LCAD compared to MCAD deficiency in mice. Pdk4 expression was unaffected in fasted Mcad<sup>-/-</sup> mice but 9 times higher in fasted Lcad<sup>-/-</sup> compared to their wild-type controls (Chapter 5 and 6). Regulation of the pyruvate dehydrogenase complex, which controls pyruvate transformation to acetyl-CoA, by PDK4 is crucial when energy demand is high 87. FFA-stimulation of Ppara is of functional importance for upregulation of Pdk4 gene expression during fasting 88 suggesting that LCAD deficiency has stronger modulatory effects on PPARα activity compared to MCAD deficiency. Importantly, ACADs can have overlapping substrate-specificities meaning that mFAO is not fully impaired when one of the dehydrogenases is defective. Acute pharmacological inhibition of mFAO, however, also did not impair de novo synthesis of G6P 89. Additionally, there are significant differences in mFAO between man and mouse, particularly with regard to substrate specificity of ACADs 90. This has to be taken into account when interpreting data obtained from mouse models of disturbed mFAO.

Decreased generation of energy via mFAO will increase the demand of glucose as source of energy and, as a consequence, decrease plasma blood glucose levels. CPT2- and VLCAD-deficient patients have been shown to have an increased respiratory exchange ratio (RER), an indicator of substrate utilization, upon exercise 91,92. This implies that impaired mFAO in these patients is compensated for by increased carbohydrate metabolism in peripheral tissues. Metabolic clearance of glucose was increased in Mcad<sup>-/-</sup> and Lcad<sup>-/-</sup> mice, which might be attributable to increased peripheral use of glucose. Peripheral glucose uptake will be further assessed by [2-<sup>14</sup>C]-deoxyglucose uptake studies in glucose-consuming organs such as adipose tissue, muscle and brain.

INTO THE ZONE

Spatial regulation of hepatic metabolism

Zonation is a major, yet infrequently considered factor in regulation of hepatic metabolism. The MIDA model used for our studies considers the liver as a single compartment without a distinct spatial separation of metabolic pathways. It does not take into account that glucose can be derived from several sources that are metabolized in different parts of the liver depending on metabolic status of the organism. In the absorptive phase, glucose is taken up by the liver where it is incorporated into fatty acids or converted to glycogen. Upon fasting, glycogen is rapidly degraded starting in periportal hepatocytes and ending in perivenous cells. In contrast to muscle, where glycogen originates mainly from glucose, hepatic glycogen can be derived from glucose and from other gluconeogenic precursors such as lactate, pyruvate and gluconeogenic amino acids. Periportal glycogen is thought to be mainly synthesized
indirectly from gluconeogenic precursors whereas perivenous glycogen is likely formed directly from glucose. This is supported by observations that glycogen is replenished in pericentral areas upon prolonged fasting (72h). Progressive fasting increases the relative contribution of gluconeogenesis to total glucose production. Gluconeogenic precursors, via G6P, are preferentially incorporated in glycogen in pericentral cells which have relatively low concentrations of G6Pase. Although it is very complicated to assess ‘zone specific’ glucose metabolism in vivo, it is of importance to consider this concept when interpreting data. Since glycerol is used as gluconeogenic precursor in our MIDA approach, additional pathways ‘feeding’ into de novo synthesis of G6P are not addressed in this model. Nevertheless, these pathways potentially add significantly to precursor formation for the gluconeogenic pathway, especially when ‘regular’ precursor formation is affected such as in disturbed mFAO. It is therefore of importance to develop methods that assess the contribution of other pathways to gluconeogenesis, e.g., lactate, pyruvate and gluconeogenic amino acids.

Using in situ hybridization, Pepck mRNA has been shown by others to be periportally localized in fed rat liver. Fasting increased the area in which Pepck was expressed. Upon isolation of periportal and pericentral hepatocytes from mice using digitonin-collagenase infusion, it has also been shown that Pepck mRNA is periportally expressed. Using a similar isolation methodology, we did not find zonation of Pepck mRNA in livers of fasted mice. QPCR analysis of laser dissected hepatocytes, however, did reveal zonation of Pepck mRNA: Expression levels were increased in periportal hepatocytes compared to pericentral hepatocytes in livers of fed mice. Pepck expression increased in both zones upon fasting, the induction being highest in pericentral hepatocytes. PEPCK protein was strongly zonated in livers of fed and insulin-infused mice. Zonation was blunted in fasted mice. These results suggested that insulin could play a role in zonation of PEPCK in the feeding to fasting response. In type 1 diabetic mice, that lack insulin, however PEPCK was still zonated suggesting that additional mechanisms regulate zonation of this enzyme in type 1 diabetes.

Insulin receptor (Ir) mRNA is homogenously expressed along the sinusoidal space. The protein, however, is mainly localized in pericentral areas of the liver, its expression being induced by glucose and venous O2 concentrations. Insulin might differentially affect carbohydrate and lipid metabolism in periportal and pericentral areas. This could partly explain the “triad” of hyperinsulinemia, hyperglycemia and hyperlipidemia which is typically seen in type 2 diabetes. The actions of insulin after its binding to IR are mediated by insulin receptor substrates (IRS) type 1 and 2 which both activate FOXO1. There is dynamic interplay between IRS1 and IRS2 in insulin signaling: IRS1 is stably expressed regardless the metabolic states of an organism whereas IRS2 expression is increased upon fasting. Interestingly, hepatic IRS1/IRS2 double knockout mice have unaffected lipid metabolism which suggests that excessive insulin signaling, rather than insulin resistance, is responsible for
hepatic dyslipidemia in type 2 diabetes. VLDL secretion is an important contributor to dyslipidemia in diabetes. Initially, VLDL secretion has been suggested to be similar in portal and central hepatocytes. However, these studies have been performed in cultured isolated hepatocytes under standardized conditions. Since portal and central hepatocytes are exposed to significantly different environments *in vivo*, it is questionable whether conclusions regarding localization of VLDL production based on these studies can be justified. Additionally, it has been shown that APOB, a structural protein of VLDL, is mainly expressed in periportal hepatocytes in mice. Insulin stimulates APOB degradation: this process is likely impaired in insulin resistance. Zonation of lipogenesis and VLDL production might explain why increased hepatic lipogenesis is not *per se* associated with increased VLDL production. Thus, in diabetes, insulin sensitivity of carbohydrate and lipid metabolism appears to be differentially affected (Figure 3). The concept of selective postreceptor hepatic insulin resistance has been supported by observations that humans with mutations in the insulin receptor have unaffected lipid levels whereas people with lipodystrophy, which is accompanied by severe insulin resistance, and subjects carrying mutations in the insulin target Akt2 have dyslipidemia. It is yet unknown if downstream targets of insulin signaling pathways are zonated across the liver acinus. Dedicated studies addressing this hypothesis will provide novel insight in zonation of hepatic insulin resistance.

![Figure 3. Zonation of hepatic (postreceptor) insulin signaling. Insulin sensitivity of carbohydrate and fatty acid metabolism is possibly differentially affected in type 2 diabetes. Additionally, bile salt synthesis has been shown to be under the control of insulin. This control has been suggested to be deficient in type 2 diabetes.](image-url)
CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Obesity is associated with increased risk to develop type 2 diabetes and CVD, two leading causes of death in Western societies. Additionally, obesity and associated health problems have significant economic impact on health care systems. Strategies to prevent and treat these metabolic derangements are therefore urgently needed. For this, better insight in regulation of interorgan as well as intraorgan metabolic pathways is of key relevance. Changes in diet and lifestyle, *i.e.*, energy intake exceeding energy output, that have taken place in the past four decades are generally accepted to underlie the obesity pandemic. The role of nutrients like glucose and specific fatty acids as molecular regulators of processes key to development of diet-related diseases has become more and more evident only over the past decade, especially after recognition of their function as ligands of transcription factors. Importantly, it should be realized that effects of nutrients on molecular signaling pathways are in general relatively mild and that food contains a variable mixture of nutrients which makes it difficult to directly associate ‘food’ with certain metabolic derangements. Moreover, long-term exposure to certain nutrients or to a combination of nutrients is often needed to facilitate changes in metabolism. Therefore, in research on food-related derangements of metabolism, data obtained from pharmacological approaches are of major importance. Pharmacological compounds that specifically activate or inhibit proteins, enzyme activities and transcription factors provide valuable insight in their roles in the system. Since the interaction between organs is complex and, to date, not reproducible in cell cultures, animal studies are still of importance in this area of research.

The complexity of interorgan communication is exemplified by the fact that activation or inhibition of one metabolic pathway, *e.g.*, by pharmacological interference or due to an inborn error of metabolism, often leads to changes in other pathways. Bile salt sequestrants are efficient in reducing cholesterol levels in patients with hypercholesterolemia. Sequestrants also improve glucose levels in type 2 diabetics. Nevertheless, we and others have shown that sequestrant treatment leads to derangements in (hepatic) triglyceride metabolism which is associated with increased risk to develop insulin resistance and CVD. Long-term consequences and severity of derangements in triglyceride metabolism upon sequestrant treatment are yet to be determined but worthwhile considering. Additionally, the consequences of bile salt sequestration might differ depending on metabolic conditions such as obesity, type 1 or type 2 diabetes.

So far, the exact mechanisms leading to hypoglycemia in mFAO defects have only partially been elucidated. It is still commonly accepted that inhibition of ß-oxidation leads to impaired gluconeogenesis. Nevertheless, we showed that rates of *de novo* synthesis of G6P are similar or only minimally decreased in several mouse models of impaired fatty acid oxidation. Likely, increased peripheral glucose consumption, *e.g.*, in muscle and brain, underlies the hypoglycemia that can occur in metaboli-
cally challenged mFAO patients although undisturbed gluconeogenesis still has to be demonstrated in human mFAO disorders. Since a reduction in hepatic gluconeogenesis appears not to be the major issue in hypoglycemic events in mFAO defects, the question might need redefinition from how come *de novo* synthesis of G6P is impaired to how come *de novo* synthesis of G6P is not upregulated to compensate for increased peripheral glucose utilization. In this respect, it is of importance to determine the contribution of gluconeogenic intermediates such as lactate, pyruvate and gluconeogenic amino acids to *de novo* synthesis of G6P in health and disease. Therefore, methods that allow *in vivo* measurement of these pathways should be developed.

Fatty liver does not necessarily lead to deranged hepatic metabolism e.g., insulin resistance or increased VLDL production. The underlying cause of a fatty liver and the location of fat in the liver are therefore likely more important determinants to develop fatty liver-associated derangements than triglyceride content *per se*. Metabolic zonation of hepatic metabolism is often disregarded in interpretation of data. Answers to unresolved problems, however, could be found in this concept. Metabolic zonation might provide significant insight in the hepatic “triad” of hyperinsulinemia, hyperglycemia and hyperlipidemia which is typically seen in type 2 diabetes. Additionally, MIDA will be differentially interpreted when considering the compartmentalization of production and breakdown of glucose and glycogen but also of fatty acids. Indeed, triglycerides are confined to perivenous areas in livers of type 2 diabetics. Although challenging, development of mouse models which lack or overexpress genes in periportal and pericentral hepatocytes and “zone-specific” targeting of pharmacological compounds will provide significant insight in the concept of metabolic zonation.

Interactions between organs can be assessed at multiple levels such as (nutri)genomics, transcriptomics, lipidomics, fluxomics, metabolomics and more. Integration of these ‘omics’ by Systems Biology approaches will provide important insight in and allows for prediction of functioning of a ‘system’ in a multitude of metabolic derangements.
REFERENCE LIST

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26. Osborne TF. CREATing a SCAP-less liver keeps SREBPs pinned in the ER membrane and prevents increased lipid synthesis in response to low cholesterol and high insulin. Genes Dev 2001;15:1873-1878.


42. Yen PM. Physiological and molecular basis of thyroid hormone action. Physiol Rev 2001;81:1097-1142.


73. Zhang X, Yeung DC, Karpisek M, Stejskal D, Zhou ZG, Liu F et al. Serum FGF21 levels are increased in obesity and are independently associated with the metabolic syndrome in humans. Diabetes 2008;57:1246-1253


84. Williamson JR, Browning ET, Scholz R, Kreisberg RA, Fritz IB. Inhibition of fatty acid stimulation of gluconeogenesis by (+)-decanoylcarnitine in perfused rat liver. Diabetes 1968;17:194-208.


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