CHAPTER 7

General Discussion

THESIS RATIONALE

The spectrum of NAFLD, which includes simple steatosis, non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis, presents a global epidemic that strongly increases the risk of cardiovascular disease [1–3]. The prevalence of NAFLD increases in parallel with obesity and associated metabolic diseases, such as type 2 diabetes (T2D) [2]. Although lifestyle modifications and bariatric surgery are effective interventions for early-stage NAFLD, there are currently no Food and Drug Administration (FDA) or European Medicines Agency (EMA)-approved pharmacological treatment options for NAFLD [4–6]. A promising class of drugs currently in phase 2 clinical trials are based on a group of hormones called fibroblast growth factors (FGFs). Especially the endocrine members of this family, FGF21 and FGF19, are of interest because of their profound effects on NAFLD alleviation in preclinical studies and clinical trials [7–9]. Our group discovered another metabolic FGF, fibroblast growth factor 1 (FGF1), which is a target gene of the lipid-sensitive transcription factor PPARγ [10]. Administration of FGF1 reduced diet-induced hepatic steatosis and inflammation in preclinical studies [11,12]. However, the metabolic pathways that are driving the hepatic lipid-lowering effects of FGF1 remain to be elucidated. While genome-wide association studies have identified several genes that increase the risk of hepatic lipid accumulation, a large part of the genetic basis of NAFLD remains unknown. A better understanding of the underlying mechanisms that contribute to the general pathophysiology of NAFLD is needed for the design and development of novel drugs. Based on our studies into the mechanism underlying the hepatic lipid-lowering actions of FGF1, we have identified several candidates that are potentially involved in this effect. In this thesis, we have studied the role of four of these candidates in hepatic function with a focus on hepatic energy metabolism. As displayed in **Figure 1**, this thesis addresses the role of O-glycosylating enzyme GalNAc-T2 in energy metabolism, the MEK-ERK signaling pathway in bile acid homeostasis and hepatotoxicity and finally, transcription factor early growth response protein 1 (EGR1) and lipid droplet-associated protein perilipin 2 (PLIN2) in hepatic lipid accumulation. Using experimental approaches, including mouse models and in vitro systems, we uncovered new functions and angles to guide future research efforts to understand hepatic energy metabolism to combat NAFLD. In the discussion section, we summarize our findings and place them in the larger context of NAFLD, metabolic effects of FGF1, and cardiometabolic health.

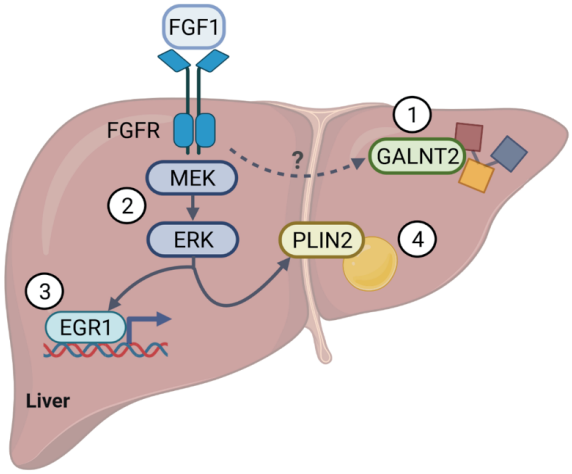

Figure 1. Outline of research chapters included in this thesis. FGF1, fibroblast growth factor 1; FGFR, fibroblast growth factor receptor; GALNT2, polypeptide N-acetylgalactosaminyltransferase 2; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; PLIN2, perilipin 2; EGR1, early growth response factor 1.

Our current understanding of the hepatic lipid-lowering effects of FGF1

Hepatic fat accumulation can occur when one or more of the following pathways are dysregulated: i) the uptake of free fatty acids, ii) hepatic *de novo* lipogenesis, iii) fatty acid oxidation in the mitochondria, iv) the secretion of triglyceride-rich very-low-density-lipoprotein (VLDL) particles [13,14]. The metabolic pathways by which FGF1 reduces hepatic steatosis remain to be elucidated. The first indication for the underlying mechanism comes from a study from our group in which FGF1 was administered to obese leptin-deficient *ob/ob* mice and the choline-deficient-amino acid defined dietary mouse model (CDAA). The CDAA model is characterized by impaired lipid catabolism due to a deficiency in lipid secretion from the liver and mitochondrial β-oxidation [15]. While FGF1 effectively reduced hepatic steatosis and inflammation in *ob/ob* mice, this was no longer observed in the CDAA model, which suggested that FGF1 mediates it hepatic lipid lowering effects via VLDL secretion and/or β-oxidation [16]. In follow-up studies, we demonstrated that FGF1 mainly stimulates triglyceride-containing VLDL secretion and mildly enhances peripheral lipid oxidation but does not affect hepatic lipogenesis and free fatty acid influx [van Zutphen *et al.,* unpublished data]. One of the mechanisms by which FGF1 potentially regulates these pathways is by enhancing the response to endoplasmic reticulum (ER) stress, *i.e.* the unfolded protein response (UPR), [van Zutphen et al., unpublished data]. We have shown both in *in vivo* and *in vitro* studies that FGF1 enhances the UPR in response to glucosamine-induced ER stress. Glucosamine induces ER stress and subsequent activation of the UPR by disrupting N-linked protein glycosylation and lipid-linked oligosaccharide

biosynthesis [17]. Glucosamine treatment of human hepatocyte cell lines induces activation of UPR markers, including GRP78 (BIP), which is further enhanced when cell lines are treated with FGF1. Conditions of NAFLD are associated with increased ER stress and a dysregulated UPR, such as the inability to adequately express hepatic spliced X-box binding protein 1 (XBP-1s), which is one of the UPR branches that regulates lipogenic genes, or failure to activate downstream recovery pathways [18–21]. While unresolved ER stress contributes to the hepatic steatosis pathophysiology, resolved ER stress by UPR activation restores ER homeostasis [21]. Therefore, restoration or manipulation of the UPR is regarded as a potential therapeutic approach for treating NAFLD. However, the mechanisms of UPR regulation and dysregulation are incompletely understood.

Despite these advances in our understanding of the biology behind the hepatic lipid-lowering effects of FGF1, the metabolic pathways and gene targets mediating this effect remain to be further elucidated. In this thesis, we have studied the individual biological function and molecular mechanisms of four candidates and their involvement in hepatic energy metabolism and hepatic function.

THE NEED TO DEVELOP NEW DRUGS TO COMBAT NAFLD

In **Chapter 1**, we provide a brief overview of energy metabolism and the central role of the liver in metabolic control. A wide variety of potential targets to treat NAFLD have been identified, yielding many experimental drugs that are currently developed and tested in clinical trials. While several of these drugs effectively reduce NAFLD/NASH in clinical trials, they often fail to be approved by FDA/EMA due to safety issues. Therefore, there is a need to develop more specific drugs with improved efficacy and less adverse effects. NAFLD is a disease that can lead to liver failure and is part of a multi-organ problem. A drug should ideally not only improve liver injury, but also glucose and insulin tolerance and adiposity. At the moment, weight loss is the only intervention that improves all histological features of NASH (steatosis, inflammation, ballooning and fibrosis) as well as glucose and insulin tolerance [5,22]. However, lifestyle intervention programs aimed at weight loss often report low attendance and adherence rates that limit health outcomes [23]. A meta-analysis of clinical trials with drugs to treat NASH reported no evidence of reduced mortality or complications of NAFLD, including cirrhosis or the requirement for liver transplantation [24]. However, most clinical trials only have a short duration of follow-up ranging from 1 month to 24 months [24]. For a progressive disease like NAFLD, a long-term follow-up is required to determine efficacy, the effect on cardiometabolic comorbidities, complications and mortality. Furthermore, NAFLD or NASH improvement should be determined by histological outcomes from biopsies taken at several time points before and after treatment [25]. Lastly, some drugs are effective in hepatic lipid lowering but cause severe adverse

effects and are therefore rejected for FDA approval. For example, the Farnesoid X receptor (FXR) agonist obeticholic acid (OCA) is effective in the treatment of NASH but also causes an increase in plasma total and low-density lipoprotein (LDL) cholesterol levels, thereby increasing the risk for developing cardiovascular disease [26]. So far, no drug has received FDA or EMA approval for the treatment of NAFLD, thus we need to find the needle in a haystack that can potentially treat NASH and cardiometabolic comorbidities without causing serious adverse effects [22]. As the etiology of NAFLD and NASH is highly complex, a better understanding of the biology and underlying molecular mechanism will help to identify novel targets.

In **Chapter 2**, we describe the current therapeutic options for NASH and review Pegbelfermin (BMS-986036), a PEGylated recombinant human fibroblast growth factor 21 (FGF21) analogue with promising effects to resolve NASH and reduce fibrosis progression without causing severe adverse effects [27,28]. We identified several issues that need to be addressed for this drug. First, liver biopsies must be taken to show short-term improvements in histology and long-term reduced progression to cirrhosis. Second, and as described above, there is no data on the effect of Pegbelfermin on long-term outcomes such as liver failure, liver transplantation and mortality. Finally, long-term adverse effects have not been reported, including loss of bone mineral density and immunogenicity. The setup of the FALCON studies, which was reported after the publication of our review, will address some of these issues. The FALCON 1 and 2 studies are phase 2b clinical trials to test the safety and efficacy of Pegbelfermin in patients with histologically-confirmed NASH with stage 3 liver fibrosis (FALCON 1; NCT03486899) or compensated cirrhosis (FALCON 2; NCT03486912) [29]. The FALCON studies have recently been completed, and the results are expected to be published shortly. Patients in this study received a once-weekly subcutaneous injection of Pegbelfermin at different doses for 48 weeks. Importantly, paired liver biopsies were taken from all patients at baseline, at week 24 for FALCON 1 and week 48 for FALCON 2, to assess histological NASH and fibrosis improvements [29]. The FALCON studies also assessed adverse effects, including bone loss and immunogenicity at six months and 14 months after the treatment. One preclinical study investigated the effect of 1-year Pegbelfermin administration on bone toxicity in monkeys and observed no effects on bone biomarkers, bone density and strength, suggesting that Pegbelfermin will likely not negatively affect bone metabolism in humans [30]. The results of the FALCON studies are expected to provide essential information which may lead to conditional FDA or EMA approval to treat NASH. However, long-term outcomes on liver failure, liver transplantation, and mortality need to be further evaluated to decide whether the benefit outweighs the possible risks of Pegbelfermin therapy.

GALNT2 IS A LIPID GENE THAT REGULATES WHOLE-BODY ENERGY HOMEOSTASIS

In **Chapter 3** of this thesis, we studied the role of GALNT2 in energy metabolism. GALNT2 encodes for GalNAc-T2, which is an enzyme that initiates mucin-type O-glycosylation at serine or threonine residues of target proteins. About 50% of the proteome undergoes O-linked glycosylation, which is vital for protein function, as it can facilitate protein trafficking, folding and stability. GALNT2 was initially shown to be associated with plasma levels of high-density lipoprotein (HDL) cholesterol and triglycerides in genome-wide association studies (GWAS) [31]. Substrates of GalNAc-T2 responsible for the effect on HDL cholesterol metabolism were found to be apolipoprotein (apo) C-III, angiopoietin-like 3 (ANGPTL3), and PLTP, which are proteins secreted by the liver [32–34]. While the regulation of HDL metabolism has been at the center of GALNT2 studies, other associations have been neglected or overlooked. We identified genetic associations between a GALNT2 gene variant (SNP rs4846914), adiposity, and body mass index in the UK Biobank population. These associations indicated a potential role for GALNT2 in whole-body energy homeostasis. In line with our findings, a recent meta-analysis study showed that the same variant was associated with increased body mass index (BMI) in a cohort of Arab individuals [35].

Following our observations in humans, we show that *Galnt2* deficient mice were smaller and gained less weight over time when fed either control or high-fat diet (HFD). While *Galnt2* deficient mice did not present an apparent liver phenotype, we noted smaller adipocytes and a marked decrease in visceral white adipose tissue (WAT) weight. Interestingly, we found reduced basal Akt-mTORC1 axis signaling, which may explain the smaller adipocytes, but a rapid and unexpectedly enhanced Akt-mTORC1 signaling in response to insulin treatment in visceral WAT derived from *Galnt2* deficient mice. In this study, we identified the insulin receptor as a novel target of GalNAc-T2, which could explain the aberrant downstream signaling. Interestingly, the *Galnt2* deficient mice also presented with a decreased respiratory exchange ratio (RER) and increased levels of plasma non-esterified fatty acid (NEFA) during the light (inactive) phase, indicating increased lipid utilization as a substrate for oxidative phosphorylation. An impaired insulin sensitivity of the visceral WAT could underlie some of the effects observed. Fat-specific insulin receptor knockout (FIRKO) mice also display lower WAT mass and smaller adipocytes without changes in whole-body glucose and insulin tolerance [36]. This suggests that insulin receptor action is impaired in visceral WAT of *Galnt2* deficient mice due to the loss of GalNAc-T2 dependent O-linked glycosylation.

Several studies reported the putative importance of other types of O-linked glycosylation for the functioning of the insulin receptor, such as O-linked β-N-acetylglucosamine (O-GlcNAc), which is partially regulated by glucose [37,38].

Elevated glucose levels leads to elevated O-GlcNAc modifications and reduced phosphorylation, which dampens insulin receptor signaling [37]. Other studies in pancreatic β cells show that partial loss of O-linked glycosylation by using benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP) leads to a constitutively active kinase activity of the insulin receptor, while treatment with glucosamine increased O-glycosylation of the insulin receptor and decreased phosphorylation of key downstream insulin signaling proteins, including Akt [39]. Taken together, these studies show that insulin receptor activity is dynamically regulated by its glycosylation status, which appears to be dependent on the metabolic state of the cell. How glycosylation of the insulin receptor by GalNAc-T2 is regulated and whether the action of GalNAc-T2 on the insulin receptor is dependent on the tissue or directly dependent on the metabolic state of the cell remains to be elucidated. More studies are required to assess whether O-linked GalNAc glycosylation sites of the insulin receptor compete or interfere with phosphorylation sites at the plasma membrane through detailed *in vitro* studies and site specific mutagenesis experiments. Another possibility is that the actions of GalNAc-T2 affect the internalization and trafficking of the insulin receptor, as insulin receptor signaling is dependent on its recycling [40,41]. Furthermore, mice lacking whole-body expression of a certain gene is a very powerful technique for the determination of gene function but the ability to create mice with a tissue-specific knockout opens new research possibilities to assess the contribution and function of a gene in a specific tissue. For example, murine knockout models of the insulin receptor in muscle, adipose tissue, liver and pancreas greatly advanced our understanding of tissue-specific insulin resistance [42]. It would be highly interesting to assess the role of *Galnt2* in adipose tissue, brain and muscle separately in future GALNT2 mouse studies.

GALNT2 deficient patients present a congenital disorder of glycosylation

Interestingly, human *GALNT2* deficiency was recently reported as a congenital disorder of glycosylation (CDG) [43]. GALNT2-CDG patients were shown to suffer from developmental delay, intellectual disability with autistic features and epilepsy [43]. These findings could be translated to the developmental problems and reduced body weight in our study in mice. Both our study and others reported embryonic lethality in mice and decreased body weight of GALNT2-deficient rodent models and cattle [43,44]. Zilmer *et al.* also showed that GALNT2 deficient rodents present abnormal behavior, something that we also observed (*i.e.* inability to get used to being handled, jumping, aggressive behavior). The studies of GALNT2 deficiency clearly display the level of complexity of the GALNT2-CDG disorder. Our study highlights that important mechanistic insights can be obtained from complex disorders.

MEK1/2 SIGNALING IS ESSENTIAL FOR THE MAINTENANCE OF NORMAL HEPATIC BILE ACID METABOLISM

The stimulatory effect of FGF1 on VLDL secretion was found to be dependent on MEK1/2 signaling in the liver, as MEK1/2 inhibition blocked FGF1's ability to increase plasma triglyceride levels [Struik et al., unpublished data]. The importance of MEK1/2 signaling in the hepatic lipid lowering of FGF1 raises the question to what extent pharmacological MEK inhibition, as used cancer treatment, could induce metabolic complications. Inhibitors directed against the RAS-MAPK pathway are known to cause adverse effects, including hepatotoxicity, diarrhea, and gastrointestinal complications, which lead to treatment interruption, morbidity, and mortality, but the underlying mechanism is unknown [45–48]. In **Chapter 4**, we evaluated short-term, *i.e.*, six days, MEK inhibition using PD0325901 in wild-type C57Bl/6 mice on liver metabolism in an attempt to a better molecular understanding of early on-target off-tumor effects.

By transcriptome analysis of the liver, we found that short-term pharmacological inhibition of MEK1/2 with PD0325901 altered the expression of over 400 genes in the liver, most of which were downregulated compared to vehicle-treated mice. Cytochrome P450, family 7, subfamily a, polypeptide 1 (*Cyp7a1*), encoding the rate-limiting enzyme responsible for converting cholesterol to bile acids, was identified as the top differentially expressed gene. Furthermore, small heterodimer protein (*Shp*), a transcriptional repressor, and other genes involved in bile acid metabolism were significantly affected by MEK inhibitor treatment. Most of these changes in gene expression were recapitulated in different human hepatocyte cell lines with PD0325901 and other MEK or BRAF inhibitors. The changes in gene expression levels also translated to differences in protein levels and were associated with changes in the composition of plasma, biliary and fecal bile acids towards a more hydrophobic bile acid content in PD0325901 treated mice compared to controls. We also observed an increase in total fecal bile acid loss, indicating an increased hepatic bile acid synthesis. Short-term PD0325901 treatment also led to changes in predictive biomarkers of early liver toxicity, but this was not accompanied by the liver or intestinal damage. In conclusion, our study shows that short-term MEK inhibition leads to profound changes in bile acid metabolism and early markers of damage, which may underlie hepatotoxicity issues later in treatment. Long-term MEK inhibition is known to result in significant hepatotoxicity and mortality. Indeed, one study reported that chronic ERK deficiency results in liver injury, cholestasis, increased plasma bile acids, and mortality [49]. Besides bile acid toxicity as discussed in Chapter 4, there can also be various other effects, as bile acids are also signaling molecules that activate multiple receptors, including the nuclear FXR and Takeda G protein-coupled receptor 5 (TGR5), which play an important role in regulating bile acid, glucose and lipid homeostasis [50–52]. While we did not observe changes in plasma glucose or

lipid levels after short-term MEK inhibition, long-term MEK inhibition and deregulation of bile acid metabolism will likely alter glucose and lipid metabolism. Hepatic FXR activation by bile acids decreases glycolysis and lipogenesis through inhibition of carbohydrate response element binding protein (ChREBP) and sterol responsive element binding protein 1 (SREBP1c), respectively [53,54]. FXR activation leads to a decrease in the production of triglyceride-rich VLDL particles [53]. FXR also promotes ApoCII and inhibits ApoC3, thereby enhancing lipoprotein lipase activity and the conversion of VLDL to intermediate-density lipoprotein (IDL) and low-density lipoprotein (LDL) [55,56]. Furthermore, MEK inhibition and the resulting increased CYP7A1 expression is also expected to decrease plasma LDL cholesterol levels as cholesterol is used to be converted to bile acids, which has been observed by one study after 14 days of PD0325901 treatment in mice [57]. Bile acids and FXR also regulate glucose metabolism, as FXR activation in enterocytes induces FGF15/19 secretion, which activates hepatic FGFR4/β-klotho, which causes increased glycogenesis and decreased glycemia [58]. Furthermore, bile acids regulate the secretion of glucagon-like peptide-1 (GLP-1), in which TGR5 activation induces while FXR activation represses GLP-1 secretion [59,60]. FXR and TGR5 activation also promote insulin secretion from pancreatic β cells, thereby also lowering blood glucose levels [61,62]. The FDA and EMA approved bile acid sequestrants as an antidiabetic drug as treatment with bile acid sequestrants deactivates FXR in intestinal L cells, thereby increasing GLP-1 secretion and decreasing intestinal glucose absorption [63]. Thus, deregulation of bile acid metabolism, as seen with MEK inhibitor treatment, may negatively affect lipid and glucose metabolism in the long term.

While FXR, TGR5 and their bile acid ligands play an important role in bile acid and energy metabolism, they have also been linked to various cancers [64–66]. Whether a certain bile acid has tumor suppressive or tumor-promoting effect depends on the type of tumor and concentration of bile acid [66]. For example, increased bile acid concentrations in the gastric juice can promote gastric intestinal metaplasia via FXR/NF-κB signaling, while hydrophobic bile acids, such as LCA, DCA and CDCA, are the main promotors of liver cancer [67–70]. Taken together, caution must also be taken concerning the tumor-promoting role of some bile acids in certain types of cancers.

MEK1/2 signaling and bile acid homeostasis in FGF1 research

CYP7A1 regulation through MEK1/2 signaling has been extensively studied for FGF19 (FG15 in mice) signaling [71]. Bile acid synthesis is tightly regulated via feedforward and feedback mechanisms involving FGF19-KLB/FGFR4 and FXR signaling, which are SHP independent and SHP dependent, respectively [72,73]. FXR stimulates FGF19 expression in the intestine, which results in activation of the FGF19-KLB/FGFR4 pathway in the liver, which seems to work in a SHP-

independent manner, as knockdown of SHP does not abolish the FGF19-mediated repression of CYP7A1 [71]. FXR signaling also stimulates SHP expression, which suppresses liver receptor homolog-1 (LRH1)-mediated stimulation of CYP7A1. FGF19-based drugs, such as NGM282, also potently suppress bile acid synthesis in humans, as reported by reduced plasma 7α-hydroxy-4-cholesteron-3-one (C4) levels, which is a marker for hepatic CYP7A1 activity [74,75]. Data from our lab using FGF1 knockout mice and FGF1/FGF2 double knockout mice studied by others indicate no differences in CYP7A1 mRNA expression, fecal bile acid excretion, or fecal bile acid profiles [76]. However, when we treated mice and human hepatocytes with recombinant FGF1, we observed a potent repression of CYP7A1 mRNA expression. Furthermore, chronic treatment with $FGF1^{\Delta HBS}$, a non-mitogenic FGF1 variant, also inhibited bile acid synthesis in mice [77]. The inhibitory effect of $FGF1^{\Delta HBS}$ on CYP7A1 expression seemed more potent than FGF19, likely because of FGF1's β-Klotho-independent binding of FGF receptors (FGFR) [77]. The same study has also shown a protective effect against induced intrahepatic cholestasis. These results indicate a similar effect of FGF1 administration on bile acid metabolism as described for FGF19, mediated through MEK1/2 signaling, which makes FGF1 a potential target for treating cholestatic liver diseases.

EGR1 REGULATES HEPATIC LIPID METABOLISM THROUGH MULTIPLE PATHWAYS

We found that the expression of the transcription factor early growth response protein 1 (EGR1) was highly induced by FGF1 treatment *in vivo*, and this effect also translated to increased protein levels of EGR1 [unpublished data]. The effect of FGF1 on EGR1, a downstream target of MEK signaling, could be recapitulated in various human hepatocyte cell lines. EGR1 is a common target of FGF1, FGF19, and FGF21, but its expression is also induced by other stimuli, including stress and other growth factors [78–80]. Apart from being a candidate potentially mediating the beneficial effects of FGFs, previous studies using whole-body *Egr1* knockout mice also suggest that EGR1 plays an important role in metabolism, including insulin sensitivity, energy homeostasis, and circadian rhythm [81–84]. Furthermore, whole-body *Egr1* knockout mice are protected against hepatic steatosis, which is likely due to its effect on adipose tissue, *i.e.,* increased energy expenditure through lipid catabolism [82]. Studies specifically focusing on the role of EGR1 in liver metabolism are currently lacking. To delineate the role of EGR1 hepatic energy metabolism and NAFLD, we developed a mouse model with somatic deletion of hepatic *Egr1* in **Chapter 5**.

Hepatic EGR1 deficient mice were created using CRISPR/Cas9-mediated gene editing and characterized after chow or HFD feeding. Interestingly, hepatic EGR1 deficiency increased hepatic triglyceride levels after HFD feeding, particularly

around the central vein. We observed increased *de novo* lipogenesis which may partially underlie the increased hepatic lipid accumulation. RNA sequencing also revealed differences in the expression of genes involved in mitochondrial and peroxisomal fatty acid β-oxidation and oxidative phosphorylation. These observations pointed towards an imbalance in fatty acid β-oxidation, by upregulation of *Cpt1a* and *Acot1*, and demand by oxidative phosphorylation, with a downregulation of genes coding for Complex I subunits, in EGR1 deficient livers. Hepatic EGR1-deficient mice also displayed increased hepatic triglyceride levels as compared to controls after overnight fasting, which is a condition that maximizes mitochondrial substrate oxidation. Furthermore, overnight fasting also increased plasma acylcarnitine levels, mainly due to an increase in acetylcarnitine (C2), suggesting alterations in fatty acid oxidation. Functional analysis using a Seahorse flux analyzer in primary hepatocytes and human HepG2 cells revealed reduced maximum oxygen consumption in EGR1 deficient cells, suggesting decreased oxidative phosphorylation. Whether the mitochondrial and peroxisomal morphology and number is affected remains to be determined using electron microscopy. We also attempted to assess the specific mitochondrial substrate utilization affected by EGR1 by performing Seahorse studies using inhibitors for the three primary substrates: long-chain fatty acids, glucose/pyruvate or glutamine. However, as the maximum mitochondrial capacity of EGR1 deficient HepG2 cells was already low, we could not further inhibit this and therefore not properly detect the affected pathway. To further investigate which mitochondrial substrate is regulated by EGR1, cellular overexpression studies are needed. We speculate that EGR1 might be involved in regulating fatty acid or glucose/pyruvate oxidation as we did observe a small decrease in maximum oxygen consumption rate in glutamine-inhibited conditions, suggesting that the glutamine pathway is still functional.

Previous studies have shown that whole-body *Egr1* knockout mice are resistant to diet-induced obesity and hepatic steatosis [82]. The reduction in hepatic steatosis was likely because of the role of EGR1 in adipose tissue, as the knockout of *Egr1* led to increased WAT energy expenditure [82]. We now show that hepatic EGR1 deficient mice are more susceptible to hepatic lipid accumulation. Thus hepatic EGR1 expression likely protects, while EGR1 expression in adipose tissue results in hepatic lipid accumulation. Therefore, we suggest that, dependent on the metabolic disturbance, tissue-specific knockout models should be taken into account as well to assess gene function in a tissue-dependent manner. This is the first study showing that hepatic EGR1 affects hepatic lipid metabolism by regulating mitochondrial fatty acid β-oxidation, respiration and *de novo* lipogenesis.

Is there a role of EGR1 in the beneficial metabolic effects of FGF1, FGF19 and FGF21?

We studied the individual contribution of EGR1 in hepatic lipid metabolism because of its potential role in facilitating the beneficial metabolic effects of FGF1, and potentially FGF19 and FGF21. As discussed, FGF1 enhances the UPR in response to ER stress. Interestingly, we observed a consistent increase in protein levels of UPR marker GRP78/BIP in livers of hepatic EGR1 deficient mice under both chow and HFD conditions [unpublished data], suggesting that EGR1 may play a role in the UPR effects of FGF1. Furthermore, EGR1 itself is upregulated by both FGF1 and glucosamine-induced ER stress [unpublished data]. It would be interesting for future studies to elucidate whether EGR1 plays a role in the increase of the UPR induced by FGF1 under ER stress conditions. FGF1 treatment has also been suggested to increase lipid oxidation as indicated by increased plasma long-chain acylcarnitine levels. However, further studies are required to elucidate whether FGF1 enhances mitochondrial fatty acid β-oxidation in the liver, as the increase in plasma long-chain acylcarnitine levels does not necessarily mean increased hepatic lipid oxidation. EGR1 might also play a role in mediating the effects of FGF19 and FGF21. Both FGF21 and FGF19 treatment in mice reduce steatosis by increasing hepatic fatty acid β-oxidation and decreasing *de novo* lipogenesis in mice, which might be (at least partially) regulated through the rapid induction of *Egr1* [85–88]. To elucidate the contribution of EGR1 in the hepatic lipid lowering effects of FGF1, FGF19 and FGF21, more in-depth studies are needed. To this purpose, hepatic EGR1 deficient mice should be fed a NAFLD-inducing diet, such as the Paigen diet, followed by a 2-week treatment with one of the FGFs, to assess the effect on steatosis and to analyze the metabolic pathways altered because of the deletion of EGR1.

PLIN2 HAS A DUAL EFFECT ON HEPATIC LIPID ACCUMULATION

Perilipin 2 (PLIN2) was also a gene found to be dynamically regulated by FGF1. We have shown that FGF1 enhanced hepatic *Plin2* gene expression acutely (*i.e.* two-fold after 6 hours *in vivo*), but decreased *Plin2* gene expression after longer-term treatment (*i.e.* after 2-5 weeks). Whether the regulation of *Plin2* gene expression is a direct or indirect effect of FGF1 treatment remains to be elucidated, as *Plin2* expression is also dependent on steatosis. Pointing at a direct effect, we observed enhanced *Plin2* gene expression and PLIN2 protein levels in various human hepatocyte cell lines after 2-6 hours of FGF1 administration [unpublished data]. Furthermore, the expression of PLIN2 follows a similar pattern as GRP78 (BIP): induction by glucosamine and enhanced expression when cells are treated with FGF1 [unpublished data]. We aimed to unravel the individual contribution of hepatic PLIN2 in hepatic lipid accumulation and ER-stress induced conditions. We used a CRISPR/Cas9 mediated somatic gene editing strategy to knockout *Plin2* in

the livers of adult mice. First, we validated the effect of a hepatocyte-specific *Plin2* knockout when mice were fed a chow diet. **Chapter 5** shows that the phenotype of hepatic PLIN2 deficient mice fed a chow diet was mostly in line with what has been reported before in liver-specific *Plin2* knockout models, namely a trend towards a decreased hepatic triglyceride content [89]. We observed that hepatic PLIN2 deficiency also led to reduced expression of UPR markers, but no changes in autophagy markers were observed when mice were fed a chow diet. Our results on autophagy markers were not in line with previous studies showing increased markers of autophagy in PLIN2 deficient livers [90]. Furthermore, experiments using primary hepatocytes isolated from hepatic PLIN2 deficient mice and controls revealed a blunted tunicamycin-induced UPR but again no clear effect on autophagy markers. The results from hepatic PLIN2 deficient mice fed a HFD were surprising. Instead of the expected decrease in hepatic lipid content in PLIN2 deficient livers, as previously reported for both whole-body and liver-specific models fed a HFD, we observed a clear increase in hepatic lipid accumulation [91–95]. It could be that germline deletion of PLIN2 reprograms the liver and therefore these mice are protected against steatosis from birth on while somatic editing induces a different program to make up for the loss of PLIN2. However, one other study using PLIN2 antisense oligonucleotide treatment also showed decreased hepatic lipid content when C57BL/6J mice were fed a HFD, which contradicts our hypothesis that germline versus somatic gene editing of PLIN2 may explain the different findings [96]. Further studies are thus needed to validate our results.

Under HFD conditions, we observed no difference in expression or levels of various UPR markers, but levels of the autophagy marker P62/SQSTM1 were doubled in PLIN2 deficient livers, indicating a reduction in autophagy. Interestingly, two other perilipin family members, namely PLIN3 and PLIN5 were upregulated both at the mRNA and protein level, which might be a compensatory mechanism for the loss of *Plin2* in HFD conditions or a consequence of the increased hepatic lipid accumulation. We also observed a marked increase in protein levels of adipose triglyceride lipase (ATGL/PNPLA2) and hormone-sensitive lipase (HSL/LIPE) in livers of hepatic PLIN2 deficient mice fed a HFD. The regulation of ATGL and HSL is essential for the balance between lipid storage and breakdown (lipolysis). ATGL catalyzes the initial step of cleaving triglycerides to diacylglycerol, followed by HSL, which hydrolyzes diacylglycerol to monoacylglycerol and fatty acid, which can be further broken down by monoglyceride lipase (MGL) to cleave monoacylglycerol to glycerol and fatty acid [97–99]. The key and predominant enzyme for of this process is ATGL, which is regulated at the transcriptional as well as post-transcriptional level. In our study, ATGL mRNA expression was only mildly upregulated, which was likely not caused by PPARα activity as its target gene expression was not generally increased, ATGL protein levels, however, were dramatically increased, indicating regulation at the post-transcriptional level. ATGL activity is known to be induced by an activator protein called comparative gene identification-58 (CGI-58) through

direct protein-protein interaction of ATGL with lipid droplet-bound CGI-58 [100,101]. Conversely, ATGL activity is inhibited by G0/G1 switch protein 2 (G0S2) [102]. Lipid droplet-associated proteins regulate CGI-58 access to ATGL by binding CGI-58 at the lipid droplet surface, which has been described for perilipin 1 (PLIN1) in adipose tissue [103,104]. The bound CGI-58 cannot activate ATGL, but phosphorylation of PLIN1 causes the dissociation of CGI-58, which is then available to activate ATGL [104]. Although the mechanism of lipolysis regulation is less well described in hepatocytes, it has been speculated that PLIN2 mediates ATGL activity by the reversible binding of CGI-58 [105,106]. In HFD fed PLIN2 deficient mice, we observed increased PLIN3 and PLIN5 mRNA expression and protein levels. PLIN3 has been shown to prevent ATGL access to the lipid droplets, while the expression of PLIN5 promotes the interaction of CGI-58 and ATGL, which both compete for interaction with PLIN5, by increasing their localization to lipid droplets [106,107]. Overexpression of ATGL normally protects against lipid accumulation and cellular triglyceride content. As we observed an strong induction of ATGL, we speculate that ATGL is accumulating but not activated, at least partially because of the increased PLIN3 and PLIN5 expression in PLIN2 deficient hepatocytes. ATGL normally has a short half-life and is degraded by the ubiquitin-proteasome pathway, but not much is known about the mechanisms by which ATGL is tagged for degradation. It could be that ATGL is only degraded after being activated and is otherwise accumulated in the cell. Fluorescent microscopy could help localize various lipid droplet-associated proteins, lipases and ATGL regulators to better understand the complex regulation of lipid droplet lipolysis. In summary, the counterintuitive increase in ATGL levels in cells with increased lipid accumulation needs further exploration and mechanistic research. The study that we describe in **Chapter 5** highlights the complexity of lipid droplet biology, and further research is required to understand how PLIN2 and other lipid droplet-associated proteins are regulated and interconnected with lipases.

FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

The understanding of biological mechanisms that contribute to metabolic dysfunction is of crucial importance for the development of new therapeutics. FGF-based drugs effectively improve dyslipidemia, hepatic injury, and hepatic steatosis. These drugs therefore represent a promising emerging class of NAFLD therapeutics of which some have already demonstrated improved health in clinical studies. FGF21- and FGF19-based drugs show promising pharmacological profiles, with decreased in liver fat and signs of improved liver histology, fibrosis and inflammation in late-stage clinical trials. Based on preclinical studies in our group, FGF1 also has therapeutic potential for treating NAFLD. The development of FGF1-based drugs, however, requires a better understanding of the metabolic pathways involved, which will not only provide information on the mechanisms of

FGF1 but also increase our understanding of the pathophysiology of NAFLD. As NAFLD and its comorbidities highly increase the risk of cardiovascular disease and mortality, clinical trials must address long-term outcomes to determine improved health. The research in this thesis highlights the perspective of four novel regulators, initially found through fibroblast growth factor 1 (FGF1) studies, in the context of (hepatic) energy metabolism. Our research contributes to our basal understanding of different regulators of hepatic energy metabolism. These findings also raise many additional questions and possibilities for future research to better understand the biology and disease pathophysiology to ultimately improve human health.

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