DOCTORAL DISSERTATION

Translational Aspects of FGF21

"From physiology to pharmacology – from mouse to man"

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Novo Nordisk A/S

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The Faculty of Health and Medical Sciences at the University of Copenhagen has accepted this dissertation for public defence for the doctoral degree in medicine. Copenhagen, 29 October 2020.

Ulla Wewer, Head of Faculty.

Opponents and review committee

Professor Mikael Rydén, Karolinska Institute (1st official opponent)

Professor Niels Jessen, Aarhus University (2nd official opponent)

Professor Birgitte Holst, University of Copenhagen (chair)

Professor Lars Edvinsson, University of Copenhagen (leader of the defence)

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"The greatest enemy of knowledge is not ignorance -

it is the illusion of knowledge"

Stephen Hawking

Preface

In 2007 three seminal papers on the impressive pharmacological effect of Fibroblast Growth Factor 21 (FGF21) in obese and diabetic rodents and monkeys piqued my interest and my FGF21 journey began. Since then more than 1700 papers on FGF21 have been published. Throughout the years I have been driven to unravel the conundrum of why a protein originally proposed to play an important role in adaptation to fasting would increase energy expenditure and insulin sensitivity in pre-clinical animal models.

This thesis is presented for obtaining a Danish doctorate degree: Dr. Med. This thesis is based on 12 years of work which I have performed at Novo Nordisk as the primary driver of FGF21 research activities. Many other tasks and projects at the company have been completed, whereas the development of FGF21 as a therapy for human disease has been challenged by our limited understanding of its physiological role, publications describing undesired side-effects in transgenic mouse models, species differences as well as less-promising data from clinical trials.

To discuss these challenges, this thesis focuses on the translational aspects of FGF21 (from physiology to pharmacology - from mice to man) and puts ten of my publications into perspective, each of them representing different translational aspects of FGF21 biology. The papers included cover the pharmacological effects of recombinant human FGF21 in different species (I-III), the physiological regulation of FGF21 and FGF21 receptors in humans in response to fasting and feeding (IV-VIII). The last two papers deal with other translational aspects of FGF21 and address overlapping pharmaco-dynamics effects of molecules (FGF21 and glucagon (IX) and FGF19 and FGF21 (X)) with distinct physiological effects. When cited in the text these publications are referenced by the Roman numerals.

I have had the privilege of working with many talented people who have supported me along my FGF21 journey. Early on, I contacted researchers at Danish universities that allowed me to gain insight into the regulation of FGF21 in humans. For this I am thankful. I also would like to thank all my co-authors for contributions to the papers. Many thanks to my colleagues at Novo Nordisk who have helped me test FGF21 in mice, pigs and monkeys and for great discussions and important contributions to the publications. I am very grateful for the fruitful collaboration I have had with Professor Bo Ahrén at Lund University. He initially provided me with plasma from the glucagon receptor KO mice to test my hypothesis on high plasma FGF21 in these mice. Dr. Bilal Omar played a key role in helping elucidate the role of FGF21 in the glucagon receptor KO mice.

I want to thank two of my former Ph.D. students, Sara Vienberg and Eva Nygaard, who have been an integral part of my FGF21 story. They have followed me for years and helped me pull data together as well as conduct part of the lab work. I also need to thank my technicians, Rikke Ingvorsen, Bettina Bonnichsen, Karen Arevad and Kirsten Vinkel Haugegaard for their excellent technical support over the years. A special thanks goes to Senior Scientist Emma Henriksson, Principal Scientist Ann Maria Kruse Hansen and Scientific Director Kirsten Raun for endless hours of FGF21 discussions and for being able to embrace "my FGF21 mind".

I have had the great fortune of meeting fantastic “FGF21 colleagues” around the world. Thanks to Dr. Alexei Kharitonenkov for his perseverance in fighting for the protein he identified on the basis of its ability to increase glucose uptake into adipocytes in 2005. I very much appreciate Professors David Mangelsdorf and Steven Kliewer at UTSW for keeping me on my toes with all their data from the genetic mouse models. We do not always agree but this has led to many good discussions. I want to thank my friends and colleagues for pushing me to write this thesis and hope I will be a better friend with more spare time in the future. I want to thank Novo Nordisk and especially my former bosses, VP Rasmus Jørgensen and CVP Jacob Steen Petersen for supporting my wish to write this thesis.

Finally, I wish to thank my incredible family, my husband Michael for endless love and support and my almost grown-up children Rasmus Emil, Jens Asger and Kristian for putting up with my many hours of reading and writing and the piles of papers lying all around our home.

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Summary

Predictive pre-clinical models are essential to drug discovery. Animal models must be carefully selected when the preclinical proof of concept and safety of a pharmaceutical target are to be established. Species homology of the target and its receptor, receptor binding and information about pharmacokinetics (PK) are important factors in translation. Despite high species homology, cross-species receptor binding and information of PK the translation of Fibroblast Growth Factor 21 (FGF21) has been challenging. The pronounced blood glucose (BG) and body weight (BW) lowering effects of FGF21 observed in mice, rats, pigs and monkeys are not observed in humans while a strong plasma lipid lowering effect is observed across species.

FGF21 belongs to the FGF19 subfamily of endocrine FGFs with metabolic rather than mitogenic effects. FGF21 binds to the short isoform of the FGF receptors 1 and 3 but only in the presence of the co-receptor beta-klotho (KLB). The FGF21 receptor complex is expressed in specific regions of the CNS and in adipose tissue. FGF21 has pleiotropic metabolic effects on energy and substrate metabolism. FGF21 treatment normalizes BG in rodent models of genetic leptin deficiency, in streptozotocin (STZ)-treated mice as well as in obese diabetic rhesus monkeys. In obese non-diabetic animal models FGF21 treatment decreases fasting plasma insulin and increases insulin sensitivity. Furthermore, FGF21 decreases BW by increasing energy expenditure (EE) in high fat-fed mice, rats and monkeys while a decrease in food intake (FI) is observed in chow-fed obese mini-pigs and spontaneously obese monkeys. FGF21 treatment also decreases plasma triglycerides and cholesterol. Putting the data together strongly suggested that a “magic bullet” for the treatment of obesity, type 2 diabetes (T2D) and dyslipidemia was found.

This initiated a search for optimized FGF21 analogues aimed for human therapy. The first clinical trials confirmed that FGF21 has metabolic activity in humans and while the lipid lowering effects were substantial only limited BG and BW lowering effects were observed in obese patients with T2D. However, FGF21 treatment decreased fasting insulin in humans. The results stood in stark contrast to the effects observed in preclinical models of diabetes and obesity. The lack of translatability may be explained by the actual physiological role of FGF21, which appears to be regulation of EE and food preferences in response to protein deficiency. This may influence FI and EE in the pre-clinical models. Furthermore, several adverse effects, with potential to limit therapeutic applications in humans, have been identified in mice overexpressing FGF21. The adverse effects which include decrease in female fertility, decrease in bone mineral density (BMD) and an increase in plasma corticosterone may, however, be linked to energy deficiency mimicking starvation in FGF21 overexpression mice. Importantly, no adverse effects on BMD nor plasma cortisol were observed obese pigs or monkeys treated with FGF21.

While the pharmacological effect of FGF21 was well established in pre-clinical models, the physiological role of FGF21 remained poorly understood. FGF21 was assigned as a fasting hormone because plasma FGF21 increases in response to an overnight fast in mice. However, plasma FGF21 is also increased in obese mice, monkeys and humans indicating a more complex biology of FGF21. Moreover, in response to fasting, insulin sensitivity and EE are decreased across species while FGF21 treatment increases insulin sensitivity and EE in mice, rats and monkeys. Thus, the proposed physiological role of FGF21 is in strong contrast to the observed pharmacological effects of FGF21. Moreover, in contrast to the significant increase in plasma FGF21 in mice in response to an overnight fast, plasma FGF21 does not increase in response to fasting for 2-3 days in healthy subjects but a significant decrease in FGF21 receptor complex expression in adipose tissue is observed, suggesting that fasting decreases FGF21 activity in humans. Plasma FGF21 is, on the other hand, increased in

response to an oral glucose tolerance test and in response to low protein feeding demonstrating a strong impact of the dietary composition on the regulation of FGF21. Thus, FGF21 is required for the metabolic adaptions in response to protein deficiency (acute and chronic) which involve changes in food preference, FI and EE, as well as glucose and lipid uptake into the adipose tissue for proper storage. FGF21-induced increases in insulin sensitivity may also involve changes in the GH/IGF-1 axis.

Other translation aspects of FGF21 include the connection between glucagon and FGF21 as well as FGF19 and FGF21. Plasma FGF21 is increased in response to exogenous glucagon treatment in mice and humans but plasma FGF21 is even more increased in mice lacking the glucagon receptor (Gcgr knockout mice (KO)). Furthermore, the anti-diabetic effects observed in the streptozotocin-treated Gcgr KO mice are partly mediated by FGF21. FGF19 is a major regulator of bile acid metabolism. However, FGF19 and FGF21 have overlapping pharmacodynamic effects (BG and BW lowering) in mice, despite distinct physiological effects. FGF15, the murine orthologue of FGF19, does however, not lower BG in diabetic mice but acts to control bile acid metabolism, supporting the notion that FGF19 cannot be used as a surrogate for FGF15 in mice.

In conclusion, the increased knowledge of the physiological role of FGF21 has facilitated our understanding of the observed pharmacodynamic effect of FGF21. It may also explain the translational gap of FGF21 as confounding factors such as the composition of the diet as well as food preference may have influenced the pre-clinical and clinical observations. The adverse effects observed in mice may be associated with lack of energy and are presumably less relevant for obese patients with T2D or non-alcoholic steatohepatitis (NASH), but additional clinical data are required to establish FGF21 efficacy and safety in humans. FGF21 analogues are currently in development for NASH and a pronounced effect on hepatic steatosis and fibrosis has been observed in mice as well as in humans. Targeting a severe disease like advanced NASH, where no treatment is available, may pave the way for clinical development of FGF21 therapies.

1 Introduction

1.1 The unmet medical need

The groundbreaking discovery of insulin by Banting and Best in 1922 [1], changed the life of people diagnosed with diabetes. Over the past almost 100 years, treatment of patients with either type 1 diabetes (T1D) or type 2 diabetes (T2D) has improved dramatically with the availability of modern insulin analogues, Glucagon-like peptide 1 (GLP-1) receptor agonists and multiple oral anti-diabetic agents. Despite, these life-changing options millions of people still suffer from diabetic complications and co-morbidities. The Western lifestyle with access to abundant food and little physical activity has greatly increased the prevalence of obesity [2] and obesity-related co-morbidities like cardiovascular diseases (CVD) and non-alcoholic steatohepatitis (NASH) [3].

Despite the increasing acknowledgment of obesity as a chronic disease, treatment options are sparse. Very few people can adhere to “diet and exercise” to control their body weight (BW) and current approved pharmacotherapy only lowers BW by 5-10%. A weight loss of up to 30% can be achieved with bariatric surgery, but the procedure is invasive and generally irreversible, hence new pharmacological treatment options are still in demand. As a consequence of their excess weight, many obese people become insulin-resistant. Initially, the pancreatic β-cell may cope with the increased demand for insulin, keeping blood glucose (BG) in control; however, if a weight loss strategy is not initiated, the insulin requirement increases, and the β-cells eventually fail leading to T2D. Body weight loss and exercise are therefore the first line of treatment for T2D, followed by oral anti-diabetic therapies. However, despite initial improvements in blood glucose control, a

progressive loss of β-cell function and mass is observed. If BW loss is not achieved and maintained exogenous insulin injections may eventually be required. Insulin therapy lowers hyperglycemia and prevents development of microvascular disease but is also associated with BW gain and risk of hypoglycemia. Therefore, treatment options that can halt progression of T2D and prevent weight gain will be very beneficial. Macro-vascular complications (cardiovascular disease (CVD)) are also highly prevalent in patients with T2D, who often have high plasma low-density lipoprotein (LDL) cholesterol (c), low high-density lipoprotein (HDL)c and high plasma triglycerides (TG). Treatment with cholesterol lowering agents is therefore initiated and can protect against cardiovascular events, if tolerated. Moreover, 80% of subjects with non-alcoholic fatty liver disease (NAFLD) have T2D. NAFLD can progress to non-alcoholic steatohepatitis (NASH) and hepatocellular carcinomas (HCC), for which there is currently no treatment available. Consequently, there is still a need for new, and improved treatment options for obesity, diabetes and the related co-morbidities.

1.2 FGF21 as a metabolic regulator

FGF21 was identified as a potential therapeutic protein in 2005 based its ability to increase glucose uptake into 3T3-L1 adipocytes [4]. In vivo, FGF21 normalizes BG in leptin deficient *ob/ob* mice and in ZDF-rats. In contrast to insulin, which induces hypoglycemia in lean rats, no hypoglycemia was observed in response to FGF21 treatment. Plasma TG levels were also decreased by FGF21 treatment in the *ob/ob* mice [4]. Similar findings were observed in obese diabetic rhesus monkeys, in which FGF21 normalized BG and decreased plasma insulin, TG and cholesterol within six weeks of treatment [5]. A small decrease in BW was also observed [5]. In diet-induced obese (DIO) mice FGF21 was furthermore shown to increase energy expenditure (EE), and a decrease in BW was also observed in spite of an increase in food intake (FI) [6, 7]. Data therefore strongly suggested that a "magic bullet" for the treatment of obesity, T2D and dyslipidemia was found. However, the translation of the effects of FGF21 has been challenging as limited BG and BW lowering effects are observed in humans [8-10].

While the metabolic actions of recombinant FGF21 were well established in several pre-clinical models in 2009, the physiological role of FGF21 remained poorly understood. FGF21 was initially suggested to play an important role in the adaptative response to fasting [11]. This was based on the observation that FGF21 is regulated by peroxisome proliferator-activated (PPAR)α [12] and that plasma FGF21 is increased in response to an overnight fast in mice [13]. Furthermore, FGF21 was shown to induce lipolysis in 3T3-L1 adipocytes [12]. However, fasting/starvation is known to decrease glucose oxidation, insulin sensitivity [14] and EE [15], which are in strong contrast to the pharmacological actions of FGF21 [6, 7, 16](III).

The plasma FGF21 concentration increases 2-3-fold in response to glucagon treatment. However, plasma FGF21 is increased 25-fold in the glucagon receptor ko mice which may explain why these mice are protected towards streptozotocin (STZ)-induced diabetes [17] (IX). As glucagon and FGF21 are regulated differently by glucose and amino acids, it is of interest to understand the connection between these two proteins. Also, FGF19 the human orthologue of FGF15, known to regulate bile acid metabolism, shows pharmacological actions (BG and BW lowering) in mice that mimics those of FGF21, while FGF15 does not lower BG in diabetic mice [18](X), suggesting that FGF19 cannot be used as a surrogate for FGF15 in mice. Therefore, a deeper understanding of the interactions between glucagon and FGF21 as well as FGF19 and FGF21 is required to understand the future potential of these hormones as human therapies.

The first part of this thesis summarizes the pharmacological effects of FGF21 observed across species and discusses the potential explanations behind the observed species differences. The second part of

the thesis challenges the view of FGF21 as a fasting factor in humans and discusses how the current insight into the physiological role FGF21 may explain many of pharmacological effects of FGF21 observed across species. The third part of the thesis discusses the current understanding of the associations between glucagon and FGF21 as well as FGF19 and FGF21. All three topics relate to the translational aspect of FGF21.

1.3 Fibroblast growth factors

1.3.1 The FGF family

The Fibroblast Growth Factor (FGF) family comprises 23 structurally related proteins which are divided into 7 sub-families according to their phylogenetic similarities [19]. The canonical FGFs (FGF1-FGF10, FGF16-FGF18) bind the FGF receptor (FGFR) and act locally in an autocrine/paracrine fashion due to their high affinity to the extracellular matrix (ECM) component heparan sulfate (HS). These FGFs are essential for embryonic development, including branching morphogenesis and limb development. Postnatally they play an important role in tissue repair and remodeling through their mitogenic and angiogenic effects [20]. The canonical FGFs signal through seven different isoforms of the FGF receptors (FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, FGFR3c and FGFR4) encoded by four genes. The seven splice variants are ubiquitously expressed, and each splice variant is expressed based on different exon splicing [21]. The FGFRs are tyrosine kinase receptors and dimerization is required for activation [22]. Furthermore, integrins can also bind the canonical FGFs and enhance their angiogenic potential [23]. Uncontrolled FGF activity is implicated in cancer development and various FGF blocking principles are in clinical development or approved for treatment of solid tumors as reviewed by Ghedini el al. [24].

1.3.2 The FGF19 subfamily, FGFR and co-receptors

FGF15/FGF19, FGF21 and FGF23 belong to the FGF19 subfamily of *endocrine* FGFs based on their atypical structure. Members of this subfamily lack the heparin binding domain and do not bind HS. This enables the endocrine FGFs to escape the cellular matrix and enter the circulation to act as hormonal-like messengers [25]. In contrast to the classical FGFs which are highly mitogenic, the endocrine FGFs are metabolic hormones [26]. The endocrine FGFs cannot bind the FGFR without the presence of a transmembrane co-receptor klotho (alfa or beta-klotho) [27, 28]. Klotho proteins consist of an extracellular domain, a single-pass transmembrane region and a short cytoplasmic tail. The co-receptors do not induce intracellular signaling and the extracellular domain serves as a docking site to facilitate the interaction between the endocrine FGFs and the FGFRs [29]. FGF19 and FGF21 both use KLB as co-receptor while FGF23 uses KL. The sequence homology of the C-terminal regions of FGF19/FGF21 and FGF23 is low, supporting distinct interactions with the two co-receptors [30]. In contrast to the FGF receptors, which are ubiquitously expressed, KL and KLB are only expressed in a subset of tissues [27, 28, 31]. KLB is highly expressed in liver, gallbladder, exocrine pancreas, white adipose tissue (WAT), brown adipose tissue (BAT) and in very specific regions of the central nervous system (CNS) [21, 32], while KL is highly expressed in the kidney. In the presence of KLB, FGF19 and FGF21 bind and signal through the short isoforms (c-isoform) of the FGF receptors 1, 2 and 3 [27, 31, 33], while only FGF19 appears to signal through the FGFR4 [28, 31]. FGFR4 is highly expressed in the hepatocytes and important for FGF19-mediated regulation of bile acids homeostasis and hepatocyte proliferation [34]. FGF23 binds and activates the FGFR2c, 3c and R4 in the presence of KL [35, 36]. Truncation of the C-terminal portion of FGF21, which binds to KLB, decreases the affinity towards the receptor complex of FGF21, while truncation of the N-terminus, which binds the FGFR, affects efficacy [30, 37]. The crystal structure of FGF21 has recently been published confirming that the C-terminal of FGF21 binds KLB while the N-terminal binds the FGFRs [38].

1.3.3 Signaling

Binding of the FGFs to the FGFRs promotes a dimerization of the receptor complex [39], which initiates an auto-phosphorylation of the receptor tyrosine residues and phosphorylation of the docking protein FGFR substrate 2-α (FRS2α). The activated FRS2α transduces intracellular FGF signaling via recruitment of several adaptor molecules like the growth factor receptor-bound protein 2 (GRB2). This initiates a cascade of signaling events including activation of the Ras/MAPK (mitogenic-activated protein kinase), phosphatidylinositol 3-kinase (PI3K)/Akt and early growth response-1 (EGR-1) pathways as reviewed by Ornitz in 2015 [40]. Currently, it is not fully understood how the canonical FGF's mitogenic signaling differs from the endocrine FGFs metabolic signaling. Dimerization strength of the FGF receptor complex, caused by differences in ligands affinities towards to receptors, is suggested to be a differentiating factor [41]. Furthermore, the tissue selective expression of the co-receptors [21] as well as the lack of co-receptor expression in dividing cells [4] appear to be contributing factors to the non-mitogenic effect of endocrine FGFs.

1.3.4 FGF15/FGF19

FGF19 and FGF15 are orthologues, but only share 52% amino acid homology [42]. The *FGF19* gene was cloned in 1999 by homology to the mouse orthologue *Fgf15* from retina [43]. The human *FGF19* gene encodes a 216 amino acid protein with a 22 amino acid signal peptide while the murine *Fgf15* gene encodes a 218-amino acids protein with a 25 amino acids signal peptide [44]. FGF19 (FGF15 in mice) is expressed in the ileal enterocytes and in the gall bladder [21]. FGF19/FGF15 is released from the enterocytes into the enterohepatic circulation, in response to bile acids via activation of the farnesoid X receptor (FXR) [45, 46]. FGF19/FGF15 regulates hepatic bile acid synthesis by inducing transcription of the small heterodimer partner (SHP) which decreases the expression of the rate limiting enzyme in bile acid metabolism, cholesterol 7 alpha-hydroxylase (CYP7A1) [44]. FGF19/FGF15 also controls refilling of bile acids into the gall bladder [47] and therefore acts opposite to cholecystokinin (CCK) [48]. Global deletion of the *Fgf15* gene in mice increases hepatic *Cyp7a1* mRNA expression, plasma bile acids and increases fecal bile acid excretion [44]. Furthermore, genetic ablation of the *Klb* gene in mice increases hepatic *Cyp7a1* mRNA expression and bile acid synthesis [49].

1.3.5 FGF21

The mouse and human *Fgf21/FGF21* genes were cloned by Nishumura et al. in 2000 [50]. The *FGF21* gene encodes a 207 amino acids polypeptide with 26 amino acid signal peptide and the mature circulating protein is a 181 amino acid protein. Mouse and human FGF21 are 80% homologous. FGF21 is highly expressed in liver and pancreas while lower expression is observed in adipose tissue and skeletal muscle across species [21, 50-54](VII). In mice FGF21 is released from the liver in response to fasting and free fatty acids (FFA) [12, 15] by activation of PPARα receptor [55]. As FFA induces the hepatic FGF21 expression, FGF21 has been suggested to be part of a negative feedback loop inhibiting lipolysis secondary to activation of PPARα by FFA [56, 57](V). Hepatic FGF21 is, however, also increased in response to high glucose via activation of carbohydrate-responsive element-binding protein (ChREBP) [58]. The FGF21 released in response to glucose has been suggested to be involved in regulation of food preferences [59] but may also increase non-insulin dependent glucose uptake in the periphery [4]. Importantly, FGF21 is released in response to insufficient amino acid supply that triggers the integrated stress response and activates the general control nonderepressible 2 (GCN2) [60]. The physiological role of FGF21 has been widely discussed, but observations in FGF21 KO mice suggest that FGF21 inhibits lipolysis and increases lipogenesis in adipose tissue [61-65]. FGF21 is required to increase FI and EE in response to protein restriction (PR) [60, 66]. FGF21 increases EE by up-regulation of uncoupling protein 1 (UCP-1) via norepinephrine

release from the sympathetic nerves innervating the adipose tissue [67]. FGF21 treated mice have furthermore a decreased preference for sucrose and alcohol [59, 68] and increase preference for protein [69]. Tissue specific deletion of the co-receptor KLB, has determined that KLB expression in neurons containing calcium/calmodulin-dependent kinase II a α (CamK2a) is important for the metabolic activity of FGF21 [70, 71]. The presence of KLB in the adipose tissue has also been shown to contribute to the acute insulin sensitizing effects of FGF21 [72, 73]. In general, adipose tissue is an important tissue target for both indirect and direct actions of FGF21 [74].

1.3.6 FGF23

The human *FGF23* gene was cloned in 2001 [75] and prior to its cloning FGF23 was known as phosphatonin [76]. The human *FGF23* gene encodes a 251 amino acid polypeptide with a 24 amino acid signal peptide. FGF23 is highly expressed in osteocytes [77] and osteoblasts [78] in response to vitamin D [79]. FGF23 is released into circulation in response to vitamin D and acts on the kidneys [35, 36]. In the proximal tubules of the kidney FGF23 decreases the expression of 1-alpha-hydroxylase (CYP27B1) to suppress formation of calcitriol (the active form of vitamin D), which in turn decreases the intestinal expression of the sodium-phosphate co-transporter preventing phosphate re-absorption [80]. Loss of FGF23 activity leads to increased phosphate levels. The FGF23 knockout (KO) mice [81], which closely resemble KL KO mice [82], have a disturbed mineral metabolism characterized by increased blood phosphate and calcium. The FGF23 KO mice are short lived, have severe growth retardation and have several aging-like features. Conversely, mice that overexpress KL have increased longevity [83].

A simplified presentation of the regulation and function of the three members of the FGF19 subfamily is given in Figure 1. FGF19/15 and FGF23 are expressed and released by activation of nuclear receptors and act in negative feedback systems ensuring tight regulation of plasma bile acids (FGF15/19) and plasma phosphate (FGF23). FGF21 is regulated by the nuclear receptor PPARα along with being strongly regulated by two other transcription factors, ChREBP and GCN2. FGF21 is regulated by several other metabolic stressors and is expressed in several tissues (liver, skeletal muscle, adipose tissue, pancreas, CNS etc.) as reviewed by Staiger, et al. [84].

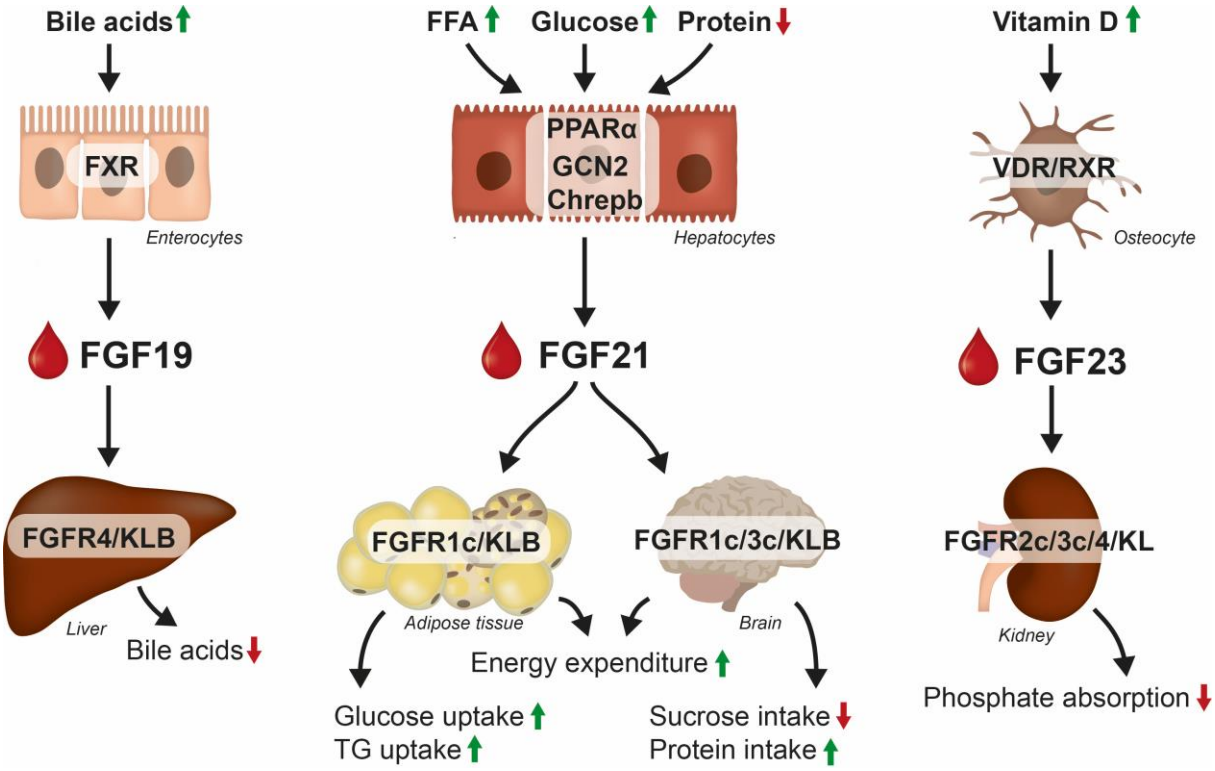

FIGURE 1 THE FGF19 SUBFAMILY

FGF19, FGF21 and FGF23 are part of the FGF19 subfamily. FGF19 is released from the enterocytes in response to bile acids and suppresses bile acids synthesis in hepatocytes. FGF21 is mainly expressed in hepatocytes in response to FFA, glucose and lack of amino acids. FGF21 acts in the CNS and in the adipose tissue to control glucose, lipid and energy metabolism, by increased glucose and TG uptake into the adipose tissue, by increasing EE and altering food preferences. FGF23 is released from the osteocytes in response to vitamin D. FGF23 suppresses production of vitamin D in the kidney which suppresses phosphate reabsorption form the kidney.

2 Part I: From Mouse to Man: FGF21 pharmacological effects in vitro and in vivo

2.1 Introduction

The pronounced metabolic effects of FGF21 observed in obese diabetic mice and non-human primates (NHP) initiated a search for optimized and stabilized FGF21 analogues allowing human therapy [8]. The initial clinical trials, however, did not confirm strong BG or BW lowering, while a pronounced lipid lowering effect was observed. With the strong anti-diabetic and anti-obesity effects observed in obese and diabetic mice, rats, pigs and monkeys, it is of high interest to understand why limited effect on BG and BW is observed in humans. Pre-clinical models are essential to narrow the translational gap to clinical testing, but the translation of results is not always straight forward. Compounds that affect EE and FI, and thereby BW, are often overestimated in small rodents with high metabolic rates, which is why several non-rodent animal models of obesity have been used to predict human efficacy of FGF21. Choice of an animal model depends of the homology of target and receptor, receptor expression if known, whereas study design and dosing depend of the expected efficacy and pharmacokinetic properties. While animal disease models of e.g. obesity and diabetes are used to predict efficacy, healthy (lean) animals are often used in toxicology programs supporting regulatory interactions and toxicity related to excess BW loss may therefore limit dosing. Part I of the thesis reviews the pre-clinical and clinical data obtained with FGF21 treatment and discusses the

potential reasons for the intra- og interspecies differences with specific focus on the hypothesis that the effect of FGF21 on EE and FI in different studies and across species may be regulated by the content and composition of macronutrients in the diet.

2.2 FGF21 and receptor homology across species

FGF21 binds the immunoglobulin-like domains (D2/D3) of FGFR1c and FGFR3c [33, 85], but only in the presence of KLB [33]. In Table 1 the sequence homology (in percent) between FGF21, FGFR1c (binding domain 2/3 (D2/D3)), FGFR3c (D2/D3) and the extracellular domain of KLB is shown for five different species. The highest homology for FGF21 and KLB is found between human and monkey. Lowest homology is observed between mice/rat and human FGF21 and KLB. The FGF receptor binding domains (D2/D3) of FGFR1c and FGFR3c are highly (≥ 99%) conserved across species.

TABLE 1 HOMOLOGY ACROSS SPECIES

% identity to human	Human	Mouse	Rat	Pig	Rhesus monkey	Cynomolgus monkey
FGF21	100	81	82	88	96	96
FGFR1c (D2/D3)	100	100	99	100*	100	100
FGFR3c (D2/D3)	100	99	99		100*	100*
Beta-Klotho (extra-cellular)	100	79	80	87	98	97

*one aa difference

2.3 In vitro potency across species

The in vitro potencies of recombinant human (rh)FGF21, are shown in Table 2. The potencies are determined as dose dependent increases in Erk signaling in human Hek293 cells overexpressing the *KLB* gene from the five species. The Hek293 cells do not express endogenous KLB but express human FGFR1c and FGFR3c and as the D2/D3 binding domains are highly conserved, the origin of the cell and therefore FGFR is less important. As seen in Table 2 the potency of rhFGF21 is higher in Hek293 cells overexpressing KLB from mice and rats, compared to cells overexpressing pig, monkey and human KLB. This in agreement with binding data showing that rhFGF21 binds with higher affinity to mouse KLB compared to human and monkeys KLB [18, 86](X). However, when tested in cells expressing endogenous levels of mouse (3T3-L1 adipocytes), rat (INS-1E insulinoma cells) or human (primary adipocytes) KLB and FGFR, respectively, no pronounced potency differences were observed between species as seen in Table 2. The high potencies of rhFGF21 observed in cells overexpressing mouse or rat KLB may therefore be an artefact of the high KLB expression level.

Recombinant human FGF21 also activates downstream signaling pathways leading to regulation of *CYP7a1* mRNA in primary cultures of rat hepatocytes [18](X) and human hepatocytes [87]. The potency of rhFGF21 in rat [18](X) and human hepatocytes (EC_{50} approx. 50 nM) [87] is decreased compared to the potency in human adipocytes (EC_{50} 0.1-5 nM). FGF21 has a higher affinity towards the FGFR1c/KLB complex compared to the FGFR3c/KLB complex [27]. Adipocytes mainly express FGFR1c [33] while FGFR3c is expressed in hepatocytes [21] and this may explain the differences in potencies between adipocytes and hepatocytes. Hepatic deletion of KLB does not affect the metabolic action of FGF21 [70] and the liver has therefore been questioned as a direct FGF21 target tissue; however, administration of rhFGF21 acutely increases downstream signaling in the liver [88-90]. The contribution of the FGFR3c/KLB complex to FGF21 activity is still questionable, as KLB agonistic antibodies, which only target the FGFR1c/KLB complex [91], display similar pharmacodynamic effects in mice [70] and monkeys [91]. In most pre-clinical studies, discussed below, rhFGF21 has been administrated by subcutaneous injection. One group has used recombinant

mouse (rm)FGF21 which showed similar metabolic effects at almost similar doses [86], and suggest that the mouse and human FGF21 have overlapping effects.

TABLE 2 IN VITRO POTENCY ACROSS SPECIES (INCLUDING DATA FROM PUBLICATION II AND X)

Species	Mouse	Rat	Pig	Monkey	Human
Hek293 KLB overexpression	0.1 nM*	0.1 nM*	2.1 nM [92]	1.8 nM*	1.1 nM [86] 2.3 nM [18]
Other cellular systems	**3T3-L1 adipocytes** 0.1 nM [86] 1 nM [4, 18]	**INS-1 cells** 1 nM [93]	ND	ND	**Primary adipocytes** 1-5 nM [94, 95] 0.6 nM [86]- 1 nM [91]

*Unpublished observation (B. Andersen). ND not determined.

2.4 FGF21 receptor expression across species

In mice the FGF21 receptor complexes (FGFR1c/KLB and FGFR3c/KLB) are highly expressed in WAT and brown adipose tissue (BAT) [21] and in specific areas of the CNS [21]. In mice KLB expression is observed in the suprachiasmatic nucleus (SCN) [32] and in the paraventricular nuclei (PVN) [96, 97] of the hypothalamus, as well as in the area postrema (AP) and the nucleus of the solitary tract (NTS) in the brainstem [32]. In NHP and in humans, only very few neurons express KLB and the main expression is observed in the midbrain and hindbrain [9, 97]. Interestingly, infants express high levels of KLB in the CNS, but a significant decrease is observed around 1 year of age [98]. In NHP and humans, the FGFR1c/KLB is expressed in the WAT [54, 57, 99](IV and V) while limited information about expression is found in BAT. Nevertheless, FGF21 has been shown to regulate downstream signaling pathways in human brown adipocytes *in vitro* [100]. The FGFR3c/KLB complex is expressed in the liver of mice, NHP and humans [9, 21, 99](IV). Differential receptor expression (location and expression level) may affect FGF21 efficacy across species, but more data are required to fully clarify if this contributes to some of the observed species differences discussed below. Furthermore, the expression of the FGF21 receptor complexes may change in pathophysiological stages and expression of the FGF21 receptor complex is, for example, decreased in WAT in obese mice, NHP and humans compared to their lean counterparts [54, 88, 99](IV). Despite an initial report of FGF21 resistance in obese mice [88], no overt FGF21 resistance has been observed in obese mice [90].

2.5 Mode of action

The current understanding of the mode of action of FGF21 is mainly based on observations in mice. The mouse is a commonly used pharmacological model, but for several reasons including its large surface to BW ratio and high metabolic rate the mouse is not always appropriate for prediction of human efficacy. The diabetic leptin deficient mouse models (*db/db* and *ob/ob* mice) are widely used. These mice are diabetic, insulin resistant and hyperphagic but the phenotype does not fully represent T2D (e.g., human T2D does not imply leptin deficiency and leptin deficient mice have extremely high fasting BG (>25 mM)) [101]. Even though mouse models may not predict human efficacy reliably, studies in mice may facilitate a mechanistic understanding of a target.

2.5.1 The anti-diabetic effect of FGF21

The metabolic action of FGF21 is mediated via the CNS [32, 70] and the adipose tissue [91]. Direct effects of FGF21 in the liver may also play a role [88-90]. Additionally, secondary effects in the pancreas and skeletal muscle may contribute to the BG lowering effect of FGF21.

Adipose tissue: "Insulin sensitivity" is a measure of insulin action in insulin-sensitive tissues usually determined by changes in the turn-over of glucose in response to insulin. Thus, improved insulin sensitivity implies decreased requirements for insulin. However, non-insulin-dependent glucose

uptake also decreases the requirement for insulin [102]. FGF21 increases glucose uptake into murine 3T3-L1 adipocytes by transcriptional up-regulation of the *Slc2a* gene encoding the glucose transporter 1 (GLUT1) protein [4, 103, 104]. The effect is independent of insulin and thus additive to the insulin effect, which increases glucose uptake by GLUT4 translocation [105]. In contrast to insulin, which can induce hypoglycemia, FGF21 does not induce hypoglycemia [4]. The lack of hypoglycemic effect of FGF21 may potentially be related to the differences in Km and Vmax (Michaelis constants) for glucose for GLUT1 and GLUT4. FGF21 also increases glucose uptake in vivo into the adipose tissue [106, 107]. Other mechanisms such as increases in plasma adiponectin [108] and decreases in inflammatory cytokines (CRP, Il6, Il1b and TNFα) in the plasma [109] have also been described to be involved in the insulin-sensitizing effect of FGF21 in DIO mice [6, 7, 110]. FGF21 has furthermore been shown to increase PPARγ activity in the adipose tissue [111]. In obese streptozotocin (STZ)-treated mice, FGF21 normalizes BG more efficaciously than in lean STZ-treated mice [112](I), supporting the important role of the adipose tissue in FGF21-mediated glucose clearance [74]. Mice with lipodystrophy are refractory to the BG lowering effect of FGF21. The anti-diabetic effect in these mice is restored by transplantation of adipose tissue and by treatment with recombinant human leptin [74]. Despite this observation of potential leptin dependent metabolic action of FGF21, FGF21 normalizes BG in leptin deficient mice [4, 18, 93](X). Other studies have shown that FGF21 lowers BG in STZ-treated mice by enhancing the function of BAT [113], and multiple studies have demonstrated that FGF21 also stimulates glucose uptake into BAT *in vivo* [72, 73, 114]. The increased requirement for glucose in the BAT may be coupled to increased mitochondrial biogenesis, UCP-1 activation and non-shivering thermogenesis [72, 103, 115]. Regarding the requirement for UCP-1 in the BG lowering effect of FGF21, one group clearly showed that the acute blood glucose lowering effect of FGF21 is impaired in the UCP-1 KO mice [107, 116], while others have shown that the BG lowering of FGF21 in maintained in the UCP-1 KO mice [117]. Furthermore, removal of BAT does not affect FGF21's ability to lower BG in mice [118, 119] but deletion of KLB in UCP-1-containing adipocytes does decrease the acute insulin sensitizing effect of FGF21 [73].

Pancreas: In pancreatic islets isolated from FGF21-treated *db/db* mice glucose-stimulated insulin release is increased [93] which indicates improved β-cell function post treatment. This may, however, be secondary to the insulin sparing effect of FGF21 discussed above. Nevertheless, FGF21 has been described to induce β-oxidation in islets, which will decrease insulin secretion [120], and FGF21 has been shown to protect towards FFA and cytokine-induced apoptosis in the rat INS-1 insulinoma cell line [93] (US2012172298). The insulin sparing function of FGF21 may also be mediated via the CNS [121] and, interestingly, FGF21 does not lower plasma insulin in vagotomized mice [122]. Finally, changes in plasma FFA and amino acids in response to FGF21 treatment may also decrease insulin secretion [123, 124]. Thus, it is unclear if FGF21 has direct effect on β-cell *in vivo*. Secretion of amylin is also decreased in response to FGF21 treatment [103] and this may potentially have a positive impact on human islets as amyloid plaques are involved in β-cell failure in humans [125].

Liver: The liver is a critical regulator of glucose, lipid and energy homeostasis. In DIO mice, FGF21 suppresses hepatic glucose production [89, 103, 106] and down-regulates genes coding for gluconeogenic enzymes, e.g., *Pepck* [6, 7, 126, 127]. Additionally, the inhibitory effect of FGF21 on lipolysis [128] decreases hepatic acetyl-CoA levels and, thereby, gluconeogenesis [129]. Furthermore, plasma glucagon is decreased in the FGF21 overexpressing transgenic (tg) mice [4] and in FGF21-treated mice [93, 103], which also will decrease hepatic glucose production. In contrast, corticosterone releasing factor (CRF) has been shown to activate the sympathetic nervous system (SNS) in response to FGF21 [130] and this could stimulate hepatic glucose production [131]. Thus, FGF21 has also been shown to increase hepatic glucose production in mice [96, 127]. Overall, controversial data on the effect of FGF21 on gluconeogenesis have been observed. The discrepancy

between the studies has been suggested to be dependent on the metabolic state (fed versus fasting). The increase in insulin sensitivity observed in FGF21 treated mice has also been described to be secondary to a decrease in ectopic fat and diacylglycerol (DAG) accumulation in liver [6, 7, 118, 128]. Gong, et al., has shown that FGF21 inhibits hepatic mammalian target of rapamycin (mTOR) activity, which increases hepatic insulin sensitivity [132] and prevents hepatic lipid accumulation [133].

Other tissues: KLB is not expressed in skeletal muscle in neither mice, monkeys nor humans [21, 54, 99, 134](IV), and the effect of FGF21 on glucose uptake in skeletal muscle [135] is believed to be secondary to the decrease in intracellular toxic lipids like DAG [118]. Yet, FGF21 has been shown to potentiate insulin-stimulated glucose transport in human skeletal muscle cells *in vitro* [136, 137]. FGF21 may also decrease plasma branched-chain amino acids (BCAA), which are associated with insulin resistance [138], and thereby increase insulin sensitivity. Lately, FGF21 has been described to lower hyperglycemia in mice via a reduction in renal glucose reabsorption through a PPARδ-mediated downregulation of sodium glucose co-transporter 2 (SGLT2) [139].

In conclusion, several mechanisms may contribute to the BG lowering effect of FGF21. However, the increase in glucose uptake into the adipose tissue does seem to play a major role as FGF21 does not lower BG in lipodystrophic mice. The insulin-independent glucose uptake will spare β-cells and lower insulin secretion and potentially protect β-cells towards exhaustion.

2.5.2 Anti-obesity effect of FGF21

Food intake and preferences: A BW lowering effect can be obtained by reducing FI (or uptake from the intestine) or by increasing EE or a combination of both. The two mechanisms are tightly correlated and a decrease in FI is associated with a with a decrease in EE. Conversely, an increase in EE increases FI. The hypothalamus is the main center controlling energy homeostasis and circulating gut-derived hormones like ghrelin [140], GLP-1 [141] and peptide YY (PYY) [142] as well as leptin released from adipocytes [143] and amylin released from β-cells [144] act on neurons in the hypothalamus to coordinate FI and EE. Plasma leptin is decreased in young FGF21 overexpressing tg mice and this may increase the expression of NPY and Agrp in the hypothalamus [6] and drive an increase in FI [4, 72]. Additionally, release of the appetite-suppressing hormone amylin is decreased [103, 144]. The hedonic system (reward/pleasure) may be also involved in FGF21-mediated FI as food preferences [59, 68] and changes in neurotransmitters (e.g. decreases in dopamine) in the nuclear accumbens have been reported in response to FGF21 treatment [68]. Interestingly, FGF21 increases the preference for protein intake in mice [69] and therefore food preferences need to be considered when the anti-obesity effects of FGF21 are studied in pre-clinical animal models.

Energy expenditure: Cold exposure increases EE by induction of non-shivering thermogenesis and heat is generated by uncoupling of the oxidative phosphorylation via UCP-1 in the adipose tissues [145]. Adipose tissue can be divided into two major depots, WAT and BAT. While WAT stores excess energy as TG, BAT is the main site of non-shivering thermogenesis. This is mediated by UCP-1 which generate heat by uncoupling the oxidative phosphorylation in this tissue. White adipose tissue stores mobilized by lipolysis are used to generate FFA for oxidation in other tissue. Specific WAT depots like inguinal adipose in rodents can also express UCP-1 in response to cold and β-3-adrenergic (β3-AR) stimuli or TZD [146]. Acute cold exposure increases plasma FGF21 [147, 148] and FGF21 activity in the CNS is required for thermoregulation in mice [148]. Also, diet-induced thermogenesis in response to high carbohydrate and fat intake involves increased activity of the SNS and BAT [149, 150]. In response to a low protein diet EE is also increased [151, 152] and FGF21 KO mice lack the ability to increase EE in response to a low protein diet [60]. FGF21 treatment increases EE and UCP-1 expression in BAT and WAT in diet-induced obese mice [6, 7]. The increase in EE is mediated by non-

shivering thermogenesis, and increases in UCP-1 activity in both WAT and BAT are observed [116]. However, UCP-1 requires FFA (lipolysis) for activity [153] and FGF21 acutely decreases lipolysis [128], but shortly after cold exposure a decrease in FFA is also observed [154] and the inhibitory effect of FGF21 on lipolysis may potentially reflect an increase in FFA utilization in BAT. The strong increase in EE in mice, regardless of increase in FI, reduces BW by 20% after two weeks of FGF21 treatment [6, 7]. The FGF21 driven thermogenesis can be blocked by the β3-AR antagonist propranolol [121] and appears to be mediated by an increase in SNS activity. Intracerebroventricular (icv) injection of FGF21 also increases SNS activity in mice [130] and in the PVN FGF21 has been shown to stimulate the release of CRF, which stimulates SNS [131]. FGF21 also enhances mitochondrial function in 3T3-L1 adipocytes through an AMP-activated protein kinase (AMPK)-Sirt1-mediated pathway which increases the rate of oxygen consumption [115] but in vivo data have shown that adipocyte AMPK is not required for FGF21 activity [155]. In mice, FGF21 has been shown to increase secretion of the chemokine C-C motif chemokine 11 (CCL11), which recruits eosinophils [156] and promotes "beiging" of WAT depots [157]. It is therefore interesting to note that FGF21 treatment also decreases BW, increases EE, lipid and glucose metabolism in mice housed at thermoneutrality (30º C for mice), although only mice housed at room temperature (RT) showed increased expression of UCP-1 in the adipose tissue [117]. This indicates that other futile cycles (e.g., Cori cycle [158], creatine energetics [159], glycolysis/gluconeogenesis [160], lipolysis/lipogenesis [6]) may be involved. Oppositely, mice lacking UCP1 (UCP-1 KO) do not increase EE in response to FGF21 [116] but BW is still reduced as FGF21 treatment also causes a decrease in FI in the UCP-1 KO mice. FGF21 has limited effect on BW and EE in *db/db* mice [4, 161-163], indicating that leptin may be required for full effects of FGF21 [164, 165]. FGF21 primarily decreases fat mass while a small, but significant, decrease in lean mass is also observed [6, 7]. Moreover, a normalization and thereby increase in physical activity was observed in FGF21 treated DIO mice and may contribute to the BW lowering effect of FGF21 [7]. FGF21 has been shown to increase lipid oxidation (respiratory quotient (Rq) decreases) [6] in DIO mice while another study showed no change in Rq in response to FGF21 treatment [7]. The ability of FGF21 to increase EE in mice is of high interest as current approved pharmacotherapy for obesity mainly decreases appetite.

2.5.3 Lipid lowering effect of FGF21

Lipids are absorbed from dietary fat or produced in the liver from carbohydrates (de novo lipogenesis (DNL)). The lipoproteins, chylomicrons and very low-density lipoproteins (VLDL)c, allow lipid transport in the blood stream. The lipids are hydrolyzed by lipoprotein lipase (LPL'ase) to release triglycerides at the target tissue, e.g., adipose tissue. Triglycerides can either be stored or used for combustion depending on need. The LDL is a cholesterol-containing lipoprotein particle. LDLc delivers cholesterol to various tissues where the LDLc is taken up by the LDL receptor. HDLc is the smallest of the lipoprotein particles and due to its high content of lipoprotein compared to cholesterol, HDLc transports cholesterol from the peripheral tissue to the liver for degradation (reverse cholesterol transport). LDLc is considered "bad cholesterol" as it can become oxidized within the walls of arteries driving atherosclerosis. Atherosclerosis is a chronic disease where high levels of plasma lipids (cholesterol and TG) and systemic inflammation cause accumulation of plaques in the arterial intima. The lipid-rich plaques have a thin fibrous cap that will eventually rupture leading to thrombosis of the narrowed vessels causing coronary heart disease (CHD), heart attack, stroke or angina.

Mice lacking FGF21 have increased plasma FFA level [62] and administration of FGF21 acutely lowers plasma FFA in mice [112, 128](I) showing that FGF21 inhibits lipolysis or increases utilization of FFA (e.g., in the adipose tissue [56]). In mice FGF21 increases the FFA transporter "cluster of differentiation 36" (CD36) and LPL'ase activity in WAT and BAT leading to an increase in uptake of

lipids [166] for utilization, storage or degradation. Lack of FGF21 causes plasma hypertriglycemia [61]. Mice lacking FGF21 also develop fatty liver [167] potentially due to an increased flux of FFA [62] and/or an impairment in hepatic β-oxidation [61]. FGF21 has been shown to increase hepatic peroxisome proliferator-activated receptor gamma coactivator 1α (Pgc-1α) expression in mice [89, 168], which induces fatty acid oxidation by increasing carnitine palmitoyltransferase (CPT1) [169]. However, FGF21 treatment decreases plasma insulin within an hour [103] which increases β-oxidation, thus the effect may be caused by a decrease in plasma insulin. Notably, overlapping phenotypes on lipid metabolism have been observed in the liver specific insulin receptor ko mice (LIRKO mice) and FGF21 treated mice [170], which indicates that the lipid-lowering effect of FGF21 is at least partly mediated by lowering of plasma insulin. The inhibitory effect of FGF21 on hepatic mTOR signaling [171] decreases DNL and increases β-oxidation [133, 172, 173]. FGF21 has also been speculated to decrease expression of proprotein convertase subtilisin/kexin type 9 (PCSK9) [174] and this mechanism may be involved in the cholesterol lowering effect of FGF21, which seems to be liver mediated [103]. Other studies have shown that FGF21 decreases hepatic cholesterol synthesis by decreasing hepatic expression of sterol regulatory element-binding proteins (SREBP)-2 [175]. In addition to the lipid-lowering effect, FGF21 increases the circulation of super oxide dismutase (SOD), glutathione (GSH) and reduces malondialdehyde [175], which all decreases oxidative stress. In mice FGF21 has also been shown to reduce Angiotensin II (Ang II) by increasing the release of angiotensin converting enzyme 2 [176]. Long term exposure to Ang II increases oxidative stress and inflammation leading to endothelial cell dysfunction, vascular smooth cell proliferation, monocyte adhesion and migration. Taken together the results how that FGF21 reduces plasms lipids and has beneficial effect on oxidative stress and therefore may protect against the development of CVD.

2.5.4 FGF21 access to CNS

Disruption of BKL using the Camk2a Cre recombinase expressed in the neurons abolish the beneficial effects of FGF21 on EE, BW, hepatic TG content and chronic insulin sensitivity [70]. Also, the FGF21-mediated decrease in sucrose and alcohol preference is abolished in the CamK2a KLB mice [68]. Furthermore, the adaptive response to PR is dependent on KLB expression in the CNS [71]. Interestingly, the effect of a BKL activation antibody (bFKB1) [91] is also abolished in the CamK2a KLB KO mice [70]. Therefore, it has been suggested that FGF21 crosses the blood brain barrier (BBB). Studies by Hsuchou et al. show that FGF21 crosses the BBB by passive diffusion [177] and FGF21 is also found the cerebrospinal fluid (CSF) [178, 179]. In contrast, another study claims that FGF21 acts on hypothalamic tanocytes (glial-like cells that line the third ventricle) and that these glial-like cells project to specific regions of the brain, as a florescent labelled FGF21 protein, remains trapped in the capillaries of the media eminence (ME) after administration [180]. Furthermore, AP and NTS are circumventricular organs (devoid of BBB) and FGF21 action in these areas can be obtained without crossing the BBB. Finally, FGF21 expression has also been observed in the CNS of rats [181] and humans [182], and a local increase in FGF21 expression in CNS may be induced by projections form the ME. The possibility of a direct effect of FGF21 on the vagus nerve, can furthermore not be ruled out, as an intact vagus nerve is required for FGF21 to lower insulin secretion [122]. It is therefore of interest to understand if other metabolic actions of FGF21 are impaired in vagotomized mice. The effect of FGF21 in the CNS is not fully understood but in addition to the stimulatory effect of FGF21 on the SNS regulation of pituitary hormones (growth hormone (GH) [92](II), adrenocorticotropic hormone (ATCH) [130] and luteinizing hormone (LH) [183]) may be direct actions of FGF21.

2.5.5 Summary mode of action

FGF21 has direct effect in the adipose tissue and in the liver [88, 90] but CNS activity is required for full metabolic activity of FGF21 [70]. FGF21 mainly lowers BG in mice by increasing glucose uptake

into adipose tissue (BAT and WAT) and by decreasing hepatic glucose production, but other insulin sensitizing mechanisms cannot be excluded. FGF21 increases SNS and EE mediated via uncoupling of the oxidative phosphorylation in the BAT. In mice, FGF21 increases FI and changes food preferences (reduced intake of sucrose and increased intake of proteins). Plasma lipids are lowered by increasing TG uptake into adipose tissue and by inhibiting lipolysis. FGF21 acutely decreases plasma insulin and hepatic mTOR activity, thereby decreasing hepatic lipid accumulation (decreasing DNL), plasma TG and cholesterol. The effects of FGF21 treatment in mice are summarized in Figure 2.

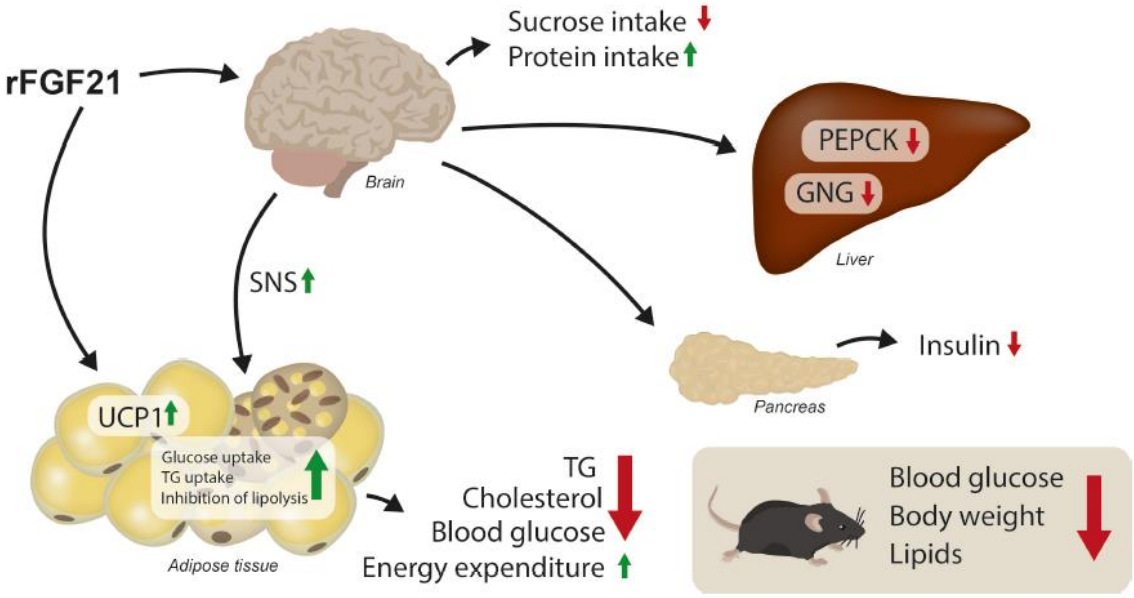

FIGURE 2 PHARMACOLOGICAL ACTION OF RECOMBINANT FGF21 IN MICE

FGF21 lowers BG in diabetic mice by increasing glucose uptake into the adipose tissue (WAT and BAT) and by decreasing hepatic glucose production. FGF21 also decreases plasma insulin, which increases hepatic β-oxidation. In DIO FGF21 increase EE by increases SNS and by increased non-shivering thermogenesis (UCP-1 dependent) in the adipose tissue. FGF21 regulates food preferences and decrease intake of sucrose and stimulates intake of protein. The TG lowering effect of FGF21 is mediated by a decrease in lipolysis and by increasing TG uptake in the adipose tissue. FGF21 inhibits hepatic mTOR activity which decreases cholesterol synthesis.

2.6 Pharmacodynamics effects of recombinant FGF21 and analogues across species

The affinity of rhFGF21 towards the rodent FGFR/KLB receptor complex may be slightly higher than that the affinity towards the pig, monkey and human receptor complex. Nevertheless, across species pharmacological effects of rhFGF21 are obtained using 0.3-3 mg/kg of rhFGF21 dosed once daily. The half-life (t½) of rhFGF21 in mice, rat, pigs and monkeys ranges from 0.6 to 4.3 hours [5, 92, 184](II). Therefore, higher doses of FGF21 are typically used in studies of mice, in which the shortest t½ is observed [5]. The pharmacological plasma exposure of FGF21 is typically magnitudes higher (10-100 µg/ml (0.5-5 nM)) [92](II) than the endogenous FGF21 plasma levels (0.1-4 ng/ml (5-200 pM)) [185]. However, based on the in vitro assays, FGF21 concentrations of more than 10 nM are required for activity. The physiological plasma levels of FGF21 in response to PR in mice are approximately 4 ng/ml equaling 200 pM [60], which is below the concentration required to activate the receptor complex *in vitro* (Table 2). However, local concentrations of FGF21 close to nerve endings and the adipocytes may be higher than the plasma values. FGF21 resistance has also been described in mice

and monkeys [88, 99] (IV) and may explain why the therapeutic plasma exposure of plasma FGF21 is magnitudes higher than endogenous plasma levels of FGF21. Furthermore, in mice the doses required to increase insulin sensitivity and to decrease plasma lipids are lower than the dose required to lower BW [7].

2.6.1 FGF21 analogues

As described above, the t½ of rhFGF21 in mice, rat, pigs and monkeys varies from 0.6 hours in mice to 4.3 hours in monkeys [5, 92, 103, 184](II) and daily injections are required to achieve pharmacological effects. Moreover, in DIO mice a chronic infusion of rhFGF21 administered by mini-pumps was shown to be more efficacious in lowering BW than the similar dose given once daily [6]. These data indicated that a FGF21 analogue with prolonged t½ is more efficacious, and less frequent dosing is also more convenient for patients. The N- and C-terminals of FGF21 are important to maintain potency and efficacy [37, 186] and as FGF21 undergoes degradation *in vivo* [86]. FGF21 analogues with stabilized N- and C-terminal have therefore been designed [95, 187]. A variety of approaches (polyethylene glycol-modified (pegylation) [184, 188-190], Fc-fusions [110, 191, 192], immunoglobulin-fusion [163], and acylations (FFA-modification to bind albumin) (WO2016102562) has been applied to increase the t½. Results from clinical trials of three FGF21 analogues have been published: 1) LY2405319: an engineered FGF21 analogue including an extra S-S bridge to enhance stability and prevent aggregation [187, 193], 2) PF-05231023: a long-acting FGF21 analogue which is comprised of two modified FGF21 molecules linked to a scaffold antibody (CovX body) [163] and 3) pegbelfermin: a PEGylated FGF21 analogue [10, 194]. The molecular weight (Mw) of the modified FGF21 analogues varies as do their plasma stability and potency; therefore, FGF21 analogue doses in mg or nmol cannot be directly compared between studies. However, the doses that have been applied in the clinical setting are comparable to the doses used in the preclinical animal models and daily dosing of 0.3 mg/kg LY2405319, which has similar Mw as FGF21, has been shown to lower plasma lipids and BW in obese patients with T2D [8].

2.6.2 Mice

FGF21 normalizes BG in leptin-deficient *ob/ob* mice [4] and in leptin receptor-deficient *db/db* mice [18, 93, 195](X). In DIO mice FGF21 increases insulin sensitivity [6, 7, 110] and the insulin-sensitizing effect of FGF21 has been observed 3 hours after dosing [103]. In obese STZ mice FGF21 normalizes BG more effectively than in lean STZ mice [112](I). In leptin-deficient mice administration of rhFGF21 only causes a slight reduction in BW and only a modest increase in EE is observed [4, 161-163] while a pronounced increase in EE is observed in FGF21 treated DIO mice [6, 7, 121]. In the high-fat fed DIO mice the increase in EE may stimulate FI [6, 7, 121], but it is unclear if the increase in FI is compensatory to an increase in EE or driven by the low protein content of the high-fat diet. No effect on BW has been shown in STZ-treated mice dosed with FGF21 [112, 119](I). FGF21 has been shown to lower total plasma cholesterol and TG in DIO mice [6, 7] and an acute plasma FFA lowering effect has been observed in *ob/ob* mice [128] and in STZ-treated mice treated with FGF21 [112](I). The pharmacodynamic (PD) effects of rhFGF21 are summarized in Table 3.

TABLE 3 PD EFFECT IN MICE (INCLUDING DATA FROM PUBLICATION I AND X)

Mice	BG	FI/EE	BW	Lipids
DIO	Increased insulin sensitivity [6, 7]	Increased EE and increase in FI [6, 7]	Decreased [6, 7]	Decrease in plasma TG and total cholesterol
Db/db	Normalization [18, 93, 161] [4]	No effect on FI [196]	Not affected [196] [4]	Decreased TG and cholesterol [196]
Ob/ob	Normalization [4, 6, 90, 162]	No effect [6] Slightly increased	Not affected/slightly decreased [6, 90, 162] [163]	TG decreased [4]
STZ treated Obese	Normalisation [112, 113]	ND	ND	FFA decreased [112]

ND not determined.

2.6.3 Rats

Fasting BG and insulin are reduced three hours after FGF21 administration to the leptin receptor-deficient Zucker Diabetic Fatty (ZDF) rats [4, 103]. An increase in plasma insulin has also been observed in FGF21 treated ZDF rats [188]. In obese Zucker rats subjected to a glucose clamp, FGF21 treatment increases glucose infusion rate and decreases hepatic glucose production [119]. Intracerebroventricular injection of FGF21 in DIO rats increases EE and followed by an increase in FI, resulting in no net effect on BW [197]. Also, an improvement in insulin sensitivity is observed in response the icv dosing of FGF21 in DIO rats [197]. A long-acting FGF21 analogue, PF-05231023, was shown to lower plasma TG in ZDF rats [188], but otherwise limited data on the effect of FGF21 have been published in rats. The PD effects of FGF21 and PF-05231023 in rats are summarized in Table 4. The limited amount of FGF21 studies in rats may be linked to lack of recombinant protein for dosing (>10 times more is required than in mice) and furthermore the expression of *Klb* mRNA in WAT in rats as measured by qPCR is low (Ct value of 34, B. Andersen unpublished) compared to mice and humans (Ct value of 30) [57] (V), potentially affecting FGF21 efficacy in rats.

TABLE 4 PD EFFECT IN RATS

Rats	BG	FI/EE	BW	Lipid
DIO	Increased insulin sensitivity [197]*	FI increased, EE increased [197]*	No effect [197]*	Plasma FFA and TG unaltered
ZDF	Normalization [4] Increased glucose infusion rate [119]**	ND [4] No effect [119]**	ND [4] No effect [119]**	ND
Zucker Obese	Glucose tolerance test OGTT improved Suppression of HGP [119]**	No effect [119]**	No effect [119]**	ND

ND: not determined *icv injection and ** data obtained with FGF21 analogue PF-05231023

2.6.4 Pigs

The beneficial effects of FGF21 on BG and EE have been described to depend on UCP-1 expression in adipose tissue [107, 116]. Humans (adults) have limited amount of BAT and the levels of BAT are negatively correlated with visceral obesity [198]. Activation of BAT by pharmacotherapy in obese subjects may be difficult. Therefore, the pig, which lacks UCP-1 [199] is an interesting model for

studying the effect of FGF21 because UCP-1-independent BW loss can be observed. However, the very severely obese Göttingen mini-pigs are neither diabetic nor dyslipidemic, and thus not a good model for BG or lipid lowering effects of FGF21 [200]. Obese female ovariectomized (OVX) mini-pigs were treated with rhFGF21 for 14 weeks. Ovariectomized pigs are used to avoid hormonal changes in FI. For the first 9½ weeks the pigs were dosed daily with 0.1 mg/kg rhFGF21 and for the last 4½ weeks the dose was increased to 0.3 mg/kg/day. After 9½ weeks no improvement was seen in insulin sensitivity as measured by an intravenous (IV)-GTT [92](II). Fasting insulin, however, was decreased [92](II). When the dose of rhFGF21 was increased to 0.3 mg/kg/day a significant improvement in IVGTT was observed. However, at this point the BW of the FGF21 treated pigs was 17% less than the vehicle group. Thus, the increases in insulin sensitivity may have been secondary to the BW lowering effect of FGF21. In the obese pigs the BW loss was driven by a decrease in FI [92](II) mimicking data in FGF21-treated UCP-1 KO mice [116]. FGF21 was unable to lower fasting plasma TG in pigs while a decrease in plasma VLDLc was observed. The PD effects of FGF21 in pigs are shown in Table 5.

TABLE 5 PD EFFECT IN PIGS (DATA FROM PUBLICATION II)

Pigs	BG	FI/EE	BW	Lipid
Obese - non diabetic [92]	Increase glucose tolerance at 0.3 mg/kg Decrease in fasting insulin	FI decreased	Decreased 17% after 14 weeks of dosing	Fasting plasma TG unchanged Decrease in VLDLc

2.6.5 Monkeys

Several of the published NHP studies have used analogues of FGF21. Two species of monkeys have been used to study the pharmacological effects of FGF21, the smaller cynomolgus monkeys [9, 201] and the larger rhesus monkey [5, 16, 110, 193](III). The first study in obese, diabetic rhesus monkeys was published in 2007 and showed a dose-dependent (30-300 µg/kg daily injection) decrease in BG [5]. A BG lowering effect was also observed in obese rhesus monkeys dosed with LY2405319 [193]. However, in one of the monkeys with severe diabetes, BG was only decreased at a dose of 50 mg/kg of LY2405319, while BW and lipids were decreased at the lower doses (3 mg/kg). In non-diabetic obese rhesus monkeys rhFGF21 improves glucose tolerance and lowers fasting insulin [16, 110](III). The effect on insulin sensitivity may be secondary to the BW lowering effect of FGF21 in these monkeys. Conversely, no significant improvement in glucose tolerance was observed in cynomolgus treated with PF-05231023 [9] despite a significant decrease in BW. FGF21 decreases BW in monkeys, but while the BW loss in DIO mice is driven by EE, the FGF21 analogue PF-05231023 lowered BW by decreasing FI in spontaneous obese cynomolgus monkeys [202]. No changes in plasma PYY or GLP-1 were observed and the effect of PF-05231023 on FI may be a direct effect of FGF21 in the CNS [68]. Conversely, in obese male and female high fat-fed rhesus monkeys, an 18% reduction in BW was observed without significant changes in FI [16](III). Thus, FGF21 is able to increase EE in NHPs in contrast to prior observations [193, 202]. The high fat diet may facilitate EE in the obese rhesus monkeys [16, 203](III), and interestingly, high fat feeding also increases EE in the FGF21 tg mice [130]. Furthermore, the amount of protein in the diet may also have influenced the findings. The spontaneously obese cynomolgus and the diabetic rhesus monkeys were fed a more protein rich diet [5, 193, 202] compared to the fat diet fed to the obese rhesus monkeys [16](III). However, more data are required to understand if the dietary composition affect EE and FI in FGF21-treated NHP. Furthermore, higher doses of FGF21 and LY2405319 decrease FI in NHP [16, 193](III) and therefore several mechanisms may be involved in the FGF21-induced BW loss in NHPs. In diabetic rhesus monkeys FGF21 decreases plasma TG by 60% and LDLc by 20% and an increase in plasma HDLc was observed [5]. In non-diabetic rhesus monkeys, only plasma VLDLc and TG were decreased by FGF21 treatment [16](III) however, these monkeys do have increased plasma cholesterol. The PD effects of FGF21 in NHP are summarized in Table 6.

TABLE 6 PD EFFECT IN MONKEYS (INCLUDING DATA FROM PUBLICATION III)

Monkeys Compounds Ref.	Doses	BG	FI/EE	BW	Lipid
Diabetic obese Rhesus rhFGF21 [5]	Dose escalating from 30-300 µg/kg for once daily for 6 weeks	Normalised BG – decrease in plasma insulin	Decrease in FI	Decreased 5 %	TG decreased LDLc decreased HDLc increased >75% reduction in VLDLc
Diabetic obese Rhesus LY2405319 [193]	Dose escalating 3, 9 and 50 mg/kg for 7 weeks	Normalised Decrease in plasma insulin	Decrease in FI	Decreased 12.5%–23.6%	TG decreased by 87% Decrease in LDLc and increase in HDLc
Obese Rhesus Monkeys rhFGF21 compared to FcFGF21 [110]	Dose-escalation Doses started at 0.1 to 3 mg/kg rhFGF21 and 0.3 mg/kg to 5 mg/kg FcFGF21 for 6 weeks	None diabetic Glucose tolerance test (OGTT) improved (only FcFGF21) Fasting insulin decreased (rhFGF21 and FcFGF21)	ND	Decreased 5% rhFGF21 12% FcFGF21	TG decreased 40% no effect on total nor cholesterol
Spontaneously obese cyno PF-05231023 [9, 202]	Dose-escalation Doses 0.1, 1 and 10 mg/kg once or twice a week for 8 weeks	None diabetic No effect on fasting BG or insulin No improvement in IVGTT	Decreased FI	Decreased 11%	TG decreased 20%
High fat fed Rhesus rhFGF21 [16]	Dose escalating 0.01-1 mg/kg once daily 12 weeks	Non-diabetic – improved in IVGTT after 12 weeks of treatment	No significant effect on FI	17-18%	TG decreased cholesterol unchanged >75% reduction in VLDLc

ND not determined

2.6.6 Humans

Several FGF21 analogues have been tested in humans. These FGF21 analogues have also been tested in mice and/or monkeys and have shown similar or more pronounced efficacy compared to rhFGF21 [163, 187, 192, 193, 202, 204-206]. LY2405319 was tested in obese patients with T2D in a multiple ascending dose (MAD) study. The patients were treated once daily with 3, 10 or 20 mg dosed by sc injections for 4 weeks [8]. LY2405319 treatment decreased BW by 1-1.5 % and lowered plasma TG by 50%, LDLc by 30% and increased HDLc by 20%. LY2405319 did not, however, normalize BG but decreased fasting plasma insulin by 40% in the 10 and 20 mg dose group. In a single ascending dose study in patients with T2D intravenous (iv) doses of PF05231023 (0.5, 1.5, 5, 15, 50, 100, 200 mg) had no effect on BG, fasting insulin or BW. It did show a 50% decrease in plasma TG at the highest dose. In a MAD study in patients with T2D, PF05231023 (iv dosing once weekly 20, 70 140 mg) lowered BW by 4-5% within 4 weeks of treatment and an improvement in plasma lipids were observed (TG decreased 50%, LDLc decreased 30% and HDLc increased 25%) [9]. A non-significant, but dose-dependent, reduction in BG (15%) and fasting insulin was observed [9]. In a subsequent study in obese patients with hypertriglyceridemia no significant effect on BW was observed after 4 weeks of treatment [201], while a dose dependent decrease in plasma TG was observed. Pegbelfermin was dosed 10 mg once-daily or 20 mg once-weekly for 16 weeks to patients with biopsy-proven NASH [194]. Approximately 40% of the patients included in the trial had T2D. No significant effect on BG or BW was observed while a small decrease in plasma lipids was observed. However, pegbelfermin decreased liver fat up to 6.8% within the 16 weeks of treatment and decreased markers of fibrosis. In a 12 weeks study, in obese T2D patients no BG or BW lowering effect of pegbelfermin was observed, but at the highest dose of 20 mg administrated once-daily, an improvement in insulin sensitivity was

observed [10]. In this study pegbelfermin lowered plasma TG by 20% [10]. The effect of FGF21 analogues in humans has recently been reviewed by Degirolamo, et al. [207] and by Struik, et al. [208]. The published clinical data of FGF21 analogues are summarized in Table 7.

TABLE 7 CLINICAL OBSERVATIONS – FGF21 ANALOGUES

Compound	Doses	Indication	BG/insulin	BW	Lipids
LY2405319 [8]	3,10 and 20 mg once daily sc for 4 weeks	Obesity and T2D	Not effect (trends toward a decrease) Insulin decreased by 50%	1-1.5 %	30% decrease in TG 30% decrease in LDLc 25 % increase in HDLc
PF-05231023 [204]	0.5 – 200mg (SAD)	T2D	No effect	No effect	50% decrease in plasma TG
PF-05231023 [9]	5-140 mg /twice a week iv) for 4 weeks	T2D	Decrease in BG (15%) and insulin	4-5% decrease	TG decreased 50%, LDLc decreased 30% and HDLc increased 25%)
PF-05231023 [201]	25, 50, 100, or 150 mg once weekly iv for 4 weeks	Obesity with hyper TG	No effect – 5% decrease in fasting insulin	Not affected	Decrease in TG (43%), increase in HDLs (28%) No effect on LDLc
Pegbelfermin [194]	10 mg daily or 20 mg weekly (for 16 weeks)	NASH	No effect	No effect	5% decrease in plasma TG
Pegbelfermin [10]	1, 5, 20 mg daily or 20 mg weekly (for 12 weeks)	Obesity and T2D	No effect on BG Increase in insulin sensitivity	No effect	20% decrease in plasma TG

2.7 PD Biomarkers across species

In addition, to the pharmacological effect on BW, BG and lipids described above, various biomarkers addressing additional PD effects have been measured across species as summarized in Table 8. Rats are excluded as limited data exist.

TABLE 8 BIOMARKERS ADDRESSING PD (DATA FROM PUBLICATION II AND III)

Species	Mouse	Pig	Monkey	Human
Insulin	Decreased [6, 7]	Decreased [92]	Decreased	40% decreased [8] 6% decreased [9, 201]
C-peptide	Decreased	Decrease [92]	Decreased [5, 16, 193]	ND
Glucagon	Decreased [93]	Decreased [92]	ND	ND
Adiponectin	Increased [108]	Increased [92]	Increased [5, 193] [16]	Increased [8, 9, 201]
Leptin	Decreased [6, 7]	ND	Decrease/unaltered [9] Decreased[193]	ND
Thyroid hormones	T3 and T4 decreased [6, 209]	T3 and T4 increased [92]	T3 decreased during washout	ND
Myostatin	ND	ND	Decreased [16]	ND

ND Not determined

Insulin/C-peptide: As discussed above FGF21 treatment decreases fasting plasma insulin without worsening of plasma glucose in all species. This indicates that FGF21 increases insulin sensitivity or non-insulin dependent glucose uptake. Plasma C-peptide is also decreased by FGF21 treatment. Moreover, the ratio between insulin and C-peptide may indicate a decrease in hepatic steatosis [92](II) as hepatic insulin internalization (clearance) is highly impacted by hepatic fat [210], whereas C-peptide is not internalized by the liver. Glucagon, a pancreatic hormone involved in regulation of blood glucose and amino acid metabolism, is decreased by FGF21 treatment in mice and pigs. In addition, to glucagon's effect on BG, glucagon also increases ureagenesis [211], and it will be interesting to observe if plasma amino acids are changed in response to FGF21 treatment.

Adiponectin: Plasma adiponectin, released from the adipose tissue, is inversely correlated to body mass index (BMI) in humans [212]. In mice adiponectin been shown to play an important role in regulation of glucose and lipid homeostasis [213], but the exact mode of action is not fully understood. FGF21 increases plasma adiponectin across species. Interestingly, in humans a potential loss of function of FGF21 identified in genome-wide association studies (GWAS) is associated with visceral obesity and insulin resistance [214], which is furthermore associated with low plasma adiponectin levels [215].

Leptin: Plasma leptin is released from adipose tissue and is during feeding (as opposed to fasting) positively correlated to BMI. Leptin plays an important role in several endocrine axes and regulates GH release, the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic–pituitary–gonadal (HPG) axis [143, 216, 217]. FGF21 treatment decreases plasma leptin in mice [4] and increases the hepatic leptin receptor expression [170], lowering the free fraction of leptin. Limited data are available regarding regulation of leptin in response to FGF21 in larger species and no data has been published in humans. In monkeys, FGF21 initially decreases plasma leptin, but plasma leptin returned to pre-treatment levels at the end of the treatment despite a decrease in BW [9]. Several of the side effects observed in mice treated with FGF21 may be related to low leptin levels and interestingly UCP-1 and leptin gene expression are reciprocally regulated in the adipose tissue [218]. Therefore, it is high interest to understand if FGF21 changes plasma leptin in higher species.

Thyroid hormones: Thyroid hormones (triiodothyronine (T3) and thyroxine (T4)) have pleiotropic effects on mitochondrial function [219] and EE. The thyroid hormones are produced and released by the thyroid glands. The inactive T4 can be converted to the active form T3 by Dionase 2, which has been shown to be increased by FGF21 treatment in the adipose tissue [6]. Active T3 increases the basal metabolic rate. Release of T3/T4 is mediated by the thyroid stimulating hormone (TSH) and a tight feedback system exists. FGF21 increases EE in mice, but a decrease in thyroid hormones has been observed [6]. The decrease in T3/T4 may be secondary to the pronounced decrease in leptin [220]. The increase in EE in FGF21 treated rats has been shown to associated with an increase in serum T3 and T4 [221]. In pigs T4 was increased in response to FGF21 treatment and may have prevented a compensatory decrease in EE normally observed in response to a decrease in FI [92](II). No significant effect on T3 and T4 was observed in FGF21 treated monkeys while a decrease in T3 was observed during the washout period [16](III). A rebound in BW is commonly observed after periods with reduced energy intake [222], and an increase in appetite together with a decrease in EE, may ensure a rapid gain of BW. More data, including TSH measurements, are required to understand the impact of FGF21 on the thyroid axis.

Myostatin: Myostatin is an inhibitor of muscle growth and therefore lack of myostatin increases muscle growth [223]. In monkeys FGF21 treatment decreased plasma myostatin [16](III), which may have prevented loss of skeletal muscle. Interestingly, during the washout period myostatin increased [16](III). The effect of FGF21 on body composition has been determined in mice, pigs and monkeys and while an improvement in body composition is observed in mice and monkeys [16](III), a loss of lean mass is observed in FGF21 treated rats (unpublished B. Andersen) and pigs [92](II) but unfortunately, plasma myostatin has not been measured in mice, rats or pigs.

In summary, several PD biomarkers are consistently altered in response to FGF21 treatment across species, indicating overlapping effects. A decrease in fasting plasma insulin and an increase in plasma adiponectin is observed in mice, pigs, monkeys and humans treated with FGF21 or analogues thereof, while more data are required to understand if leptin, thyroid hormones and myostatin are similarly affected by FGF21 treatment across species. Other PD biomarkers addressing the anti-oxidative effect (e.g., GSH) of FGF21 would be beneficial to include in future studies.

2.8 Learning from genetic models

The use of pre-clinical species to predict human efficacy has been challenging for FGF21, but within the last few years, several GWAS on FGF21 and KLB have been published and revealed important information linking the observed effect in the FGF21 KO mice to clinical observations.

2.9 FGF21 overexpressing transgenic mice

Overexpression of FGF21 in the liver in mice results in a phenotype that resembles mice treated with FGF21. The FGF21 tg mice have increased EE and are lean despite an increase in FI (FI/BW) [4, 12, 89]. However, another study showed that the FGF21 tg mice have increased fat mass [224]. The FGF21 tg mice have decreased plasma insulin, lower fasting plasma glucose [4, 72] and improved glucose tolerance [225]. The decrease in plasma insulin allowing hepatic β-oxidation [226] and β-hydroxybutyrate (BHB) formation is increased in the fed state in the FGF21 tg mice [12, 168]. The FGF21 tg mice have lower plasma FFA, TG and cholesterol compared to WT mice [4, 12, 72, 224]. The FGF21 tg mice are furthermore protected against development of hepatic steatosis [4, 12, 224] and HCC [227]. The FGF21 tg mice have increased longevity linked to a decrease in plasma IGF-1 and the linear growth of the mice is furthermore impaired [225]. The FGF21 tg mice have low preference for alcohol and sucrose [68] and increased water intake [228].

2.9.1 FGF21 knockout mice

The FGF21 KO mice [61, 64, 229, 230] have decreased thermogenic ability (decreased browning of adipose tissue) [32] and are therefore more sensitive to cold exposure than WT mice [67]. In addition, FGF21 KO mice lack the ability to expand subcutaneous fat [231] potentially due to decreased PPARγ expression in adipose tissue [61]. The FGF21 KO mice have also been reported to be insulin resistant [232] and in response to an intraperitoneal (IP) GTT, the glucose excursion rate is increased [61]. The FGF21 KO mice were initially described to have an impaired response to fasting (gluconeogenesis and ketogenesis) [168], but these findings were not confirmed by other groups [62, 233]. Nevertheless, the FGF21 KO mice have higher plasma insulin, thus a decrease in hepatic glucose production may be expected [234]. In response to fasting [233], ketogenic diet (KD) [61], high fat diet (HFD) [227], alcohol [235] and PR [236], the FGF21 KO mice are prone to develop liver steatosis. Liver weight is also increased in the basal state in the FGF21 KO mice [62] and the KO mice have increased plasma TG [63]. The accumulation of hepatic fat in the FGF21 KO may be associated with increased flux of FFA from the adipose tissue [62], but could also be caused by a reduction in hepatic β-oxidation due to higher plasma insulin [234]. Hepatic FGF21 is strongly induced by PR, and the metabolic adaptations to protein deficiency are abolished in FGF21 KO mice [66]. Furthermore, FGF21 KO mice eat more simple sugars compared to WT mice, suggesting a major role of FGF21 in control of macronutrients [59]. Finally, the FGF21 KO mice are prone to Ang II-induced hypertension [176].

2.9.2 KLB knockout mice

The insulin sensitizing, BG and BW lowering effects of FGF21 are lost when the required co-receptor KLB is globally deleted [72]. Nevertheless, the global KLB KO mice are surprisingly resistant towards HFD-induced obesity [237]. However, KLB is, as described, also a co-receptor for the FGF19/FGF15 system, and therefore mice lacking FGF15 activity have increased plasma bile acids [34, 238]. The high plasma bile acids found in the global KLB KO mice may increase EE by activation of G-protein-coupled bile acid receptor (TGR5), which increases EE and GLP-1 release [237]. Therefore, tissue-specific silencing of KLB is required to study the contribution of KLB-expressing tissues to the metabolic actions of FGF21. Disruption of KLB, using the Camk2a Cre recombinase expressed in the CNS, abolishes the beneficial effects of FGF21 on EE, BW and chronic insulin sensitivity [70]. Also, the

FGF21-mediated decrease in sucrose and alcohol preference is abolished in the CamK2a KLB KO mice [68]. Furthermore, the adaptive response to PR is dependent on KLB expression in the CNS [71]. However, KLB in the adipose tissue also contributes to the insulin-sensitizing effect of FGF21[72] and mice lacking adipose tissue are refractory to the metabolic benefit of FGF21 [103].

2.9.3 Polymorphisms in FGF21

Two independent studies in humans have shown that single nucleotide polymorphisms (SNPs) in the FGF21 locus are associated with changes in intake of macronutrients. The two alleles rs838133 [239] and rs838145 are both associated with higher carbohydrate intake [240] and therefore, potentially, also with a loss of FGF21 function. In the Danish Inter99 cohort rs838133 was furthermore linked to an increased consumption of candy [241] and decreased fat and protein intake [240, 241]. The effect of FGF21 on food preference was later confirmed in a meta-analysis including up to 123,000 individuals [242]. A GWAS from the UK Biobank (>450,000 individuals) showed that the common rs838133 allele also is associated with insulin resistance, higher blood pressure (PB) and a higher waist-to-hip ratio despite a lower total body-fat percentage [214]. Nevertheless, the effect of the rs838133 allele on these parameters is extremely small (0.33 mm Hg in PB and a 1 mm difference in hip circumference), but the effect sizes of common genetic variants does not predict the potential efficacy of a target [214]. Notably, subjects with a high hip-to-waist ratio have low plasma adiponectin [215], which is also found in the FGF21 KO. The inverse correlation between adiponectin and fasting insulin, HOMA-IR, triglyceride, systolic and diastolic BP [215, 243] potentially links adiponectin to FGF21 biology [108]. The FGF21 rs838133 allele was, however, not associated with fasting plasma glucose, but was associated with higher LDLc and higher gamma-glutamyl transpeptidase (GGT) levels [214].

2.9.4 Polymorphisms in KLB

In a Silesian and Swiss population study, SNPs in KLB have been associated with BMI [244] and accelerated colon transit [245]. The latter, presumably due to change in bile acid metabolism caused by a decrease in FGF19 activity as previously discussed. Furthermore, in a meta-analysis including more than 105,000 individuals, a locus in KLB was associated with increased alcohol consumption. A common SNP in the KLB gene (rs2608819) has been associated with a reduction of KLB expression in the adipose tissue and a higher BMI, potentially linking FGF21 activity to EE in humans [244]. Mutations in the KLB gene have also been associated with congenital hypogonadotropic hypogonadism [180], a rare disease with absent or partial puberty and infertility caused by deficiency of gonadotropin-releasing hormone (GnRH). Therefore, loss of KLB function, affects both FGF19 and FGF21 activity in humans. The former results in changes in bile acid metabolism and in colon transit, while the alcohol intake associated with SNPs in KLB is speculated to be caused by lack of FGF21 function.

2.9.5 Genetic consequences in mice versus humans

Available genetic phenotypes related to loss of FGF21 function in mice and in humans are shown in Table 9. The most striking similarity is the sucrose and alcohol preferences found in FGF21 KO mice [59], KLB CamK2a KO mice [68] and in humans carrying the rs838133 allele [239] who have higher carbohydrate intake [241], while humans carrying a mutation in KLB have higher alcohol consumption [246]. Notably, plasma FGF21 is increased in response to an OGTT [247](VIII) and after alcohol consumption [248] and the acute increase in plasma FGF21 may prevent further intake of sucrose and alcohol. This effect may be lost in individuals with the described SNPs.

The decrease in total body-fat percentage in individuals with the rs838133 allele [214] correlates with the decreased ability to store fat in the subcutaneous adipose tissue also described in the FGF21

KO mice [65]. Finally, FGF21 KO mice are prone to develop hepatic steatosis, NASH and HCC [227] and it is, therefore, of great interest to understand if humans with decreased FGF21 activity are at greater risk of developing NASH. The genetic data strongly suggest overlapping phenotypes related the loss of FGF21 activity in mouse and human. Thus, mice can be used to predict FGF21 efficacy in humans if the mice studies are conducted under the right conditions (housing temperature and available food/choice of food).

Table 9 LACK-OF-FUNCTION MUTATIONS IN MICE VERSUS HUMANS

	Mouse	Human
FGF21	Global FGF21 KO	Potential loss of function SNP rs838133* rs2608819**
Reported effects	Decrease SC fat [65] Insulin resistance [232] Ang II -induced hypertension [176] FGF21 ko have preference for sucrose [59] Decreased EE [32]	Visceral obesity*[214] Decreased fat %**[214] Insulin resistance**[214] Hypertension**[214] Increased consumption of candy* [241] Increase in BMI** [244]
KLB	Camk2a KO	Potential loss of function
Reported effect	Mice with KLB CNS KLB ko have increased alcohol and sucrose consumption [68]	Linked to increased alcohol consumption [246]

2.10 Translational aspects of pharmacology from mice to man

In summary, as shown in Table 10 the lipid-lowering effects of FGF21 have been translated from mice [6, 7] to humans with or without T2D [8-10, 194], while inconsistent effects on BW and BG have been observed. However, fasting plasma insulin was decreased in T2D treated with LY2405319 for 4 weeks and 12 weeks of pegbelfermin increased insulin sensitivity in T2D. Furthermore, plasma adiponectin is increased in all species treated with FGF21. The anti-obesity effect observed in high fat-fed mice, rats and monkeys is driven by an increase in EE, while FGF21 decreases FI in the chow-fed obese cynomolgus monkeys, in diabetic rhesus monkeys and in pigs. It is currently not known if the BW lowering effect observed in humans treated with FGF21 analogues [8, 9] is driven by a decrease in FI or an increase in EE. Interestingly, increases in appetite is a common reported side effect in humans treated with FGF21 analogues and may be secondary to changes in EE, yet this must be clarified. FGF21 has been described to affect food preference in mice [59, 68, 69, 71] and it will be of great interest to observe if humans treated with FGF21 analogues experience changes in behavior and food preferences. The genetic evidence from humans (loss of function) indicate the FGF21 may not decrease BW, but may induce fat storage in the subcutaneous depot, thus removing ectopic fat accumulation from liver and skeletal muscle and increasing insulin sensitivity.

TABLE 10 PHARMACODYNAMIC EFFECT OF FGF21 ACROSS SPECIES (INCLUDING DATA FROM PUBLICATION II AND III)

	BG	BW	FI/EE	Lipid-lowering
Mice	Normalised	Decreased	EE increased (high fat fed mice)	TG and choletserol decreased
Rat	Normalised	Decreased	EE increased (high fat fed rats)	TG decreased
Pigs	Insulin sensitising effect	Decreased	FI decreased (Chow fed)	TG unaltered
Monkeys	Normalised Insulin sensitizing effect	Decreased	EE increased (high fat fed monkeys) FI decreased (Chow fed)	TG decreased LDLc decreased HDLc increased
Humans	Insulin sensitizing Trends towards BG lowering effect	Decreased unaltered	Unknown	TG decreased LDLc decreased HDLc increased

2.10.1 Lack of BG lowering effect in humans

One of the first potential explanation for why FGF21 analogues are unable to normalize BG in humans may relate to the low amount of BAT [249] which is significantly lower in adults [249, 250] compared to mice housed at RT [251]. However, FGF21 has also been shown to induce browning of the WAT [6, 157] which also increases glucose uptake into the adipose tissue. This may, however, require a longer treatment period and may involve post-prandial activation of SNS which may be impaired in obese subject with T2D [252]. KLB is furthermore only expressed in a few neurons in adults [97] compared to mice [183], but, interestingly, newborns have high KLB expression in the CNS [98] indicating an important role of FGF21 in non-shivering thermogenesis in infants [253]. If the BG lowering effect of FGF21 requires CNS activity of FGF21 then this may be impaired in humans compared to mice. Secondly, the potentiating effect of FGF21 on PPARγ activity in the adipose tissue [111] may also require longer treatment. In response to pioglitazone (a PPARγ agonist) glycosylated haemoglobin (HbA1c) is decreased 0.8% after 16 weeks of treatment in patients with T2D [254]. The longest published human study using an FGF21 analogue (pegbelfermin) was a 16-week study in patients with NASH (40% with T2D) and no effect on glycosylated HbA1c was observed. However, pegbelfermin is, based on its lipid lowering effect, a weak FGF21 agonist, thus trials of longer duration with more potent FGF21 analogues may decrease BG in humans. Moreover, the PPARα agonist fenofibrate has anti-diabetic effect in mice [255] while no BG lowering of fenofibrate is observed in humans. Insulin induces glucose uptake into skeletal muscle and adipose tissue, while FGF21 only regulates *Slc2a* expression in adipocytes. Therefore, lack of glucose uptake into skeletal muscle may also contribute to a low BG lowering effect in humans, especially if BAT drives major uptake of glucose uptake in mice. Interestingly, a very encouraging observation is that a 43% improvement of glucose infusion rate was observed in T2D during a clamp study after 10 days of cold exposure [256]. These results exceed what is observed in response to long-term exercise training.

The identification of mTOR as a regulatory node in FGF21 signaling in adipocytes is also of interest [257] as a common side effect of the mTOR inhibitor rapamycin (sirolimus), an immunosuppressive agent used to prevent rejection of organ transplant, is insulin resistance [258] and risk of T2D development [259]. If the mechanism by which sirolimus increases insulin resistance is mediated by lack of glucose uptake into adipose tissue in humans, FGF21 in time also increase insulin sensitivity in humans. Finally, it is remarkable that FGF21 lowers BG in mice as several hormones (glucocorticoids [32], GH [12] and catecholamine [121, 130]) known to cause insulin resistance are induced by FGF21

treatment. Glucocorticoids increase hepatic gluconeogenesis, induce visceral obesity and cause insulin resistance [260]. GH increases lipolysis and causes insulin resistance [261] and catecholamines decrease insulin release [262]. Interestingly, FGF19, a closely-related FGF21 analogue in mice [18](X), has been shown to normalize BG in STZ-treated mice by decreasing glucocorticoid release [263]. More studies are required to understand if GH, glucocorticoids and catecholamines are affected by FGF21 treatment in obese and diabetic animal models. Plasma cortisol was not increased in obese pigs [92] (II) and monkeys [16](III) treated with FGF21. Elevation in plasma GH decreases glucose oxidation and insulin sensitivity and the limited effect of FGF21 treatment on BG in humans may relate to the observed discrepancies in regulation of the GH/IGF-1 between pre-clinical models [224] [16, 92](II and III) and humans [9]. In mice [12], pigs [92](II) and monkeys [16](III) a decrease in total plasma IGF-1 is observed, while one study measured an increase in total IGF-1 in humans treated with PF-05231023. In humans, changes in food preference towards a more protein-rich diet could also affect plasma IGF-1 [264] and insulin sensitivity [265]. Interestingly, following 10 days of a low protein diet BG is decreased and insulin sensitivity increased in T1D patients [266]. This is potentially mediated by FGF21 [60] support FGF21-mediated glucose uptake in human. Patients with T1D have decreased plasma FGF21 [267] but recent study did not find any correlations between plasma FGF21 and BG. However, T1D patients with retinopathy had significantly lower plasma FGF21 levels compared to healthy individual [268]. Metformin, a commonly used anti-diabetes agent, has furthermore been shown to increase FGF21 secretion in hepatocytes [269], and part or the anti-diabetic effect of metformin could be mediated by FGF21. However, no association between the human SNPs affecting FGF21 activity, and T2D has been described [214]. In summary, it is too early to conclude on the potential of FGF21 to lower BG in humans, but FGF21 may potentially be used in combination with insulin to increase insulin sensitivity and reduce the risk of hypoglycemia.

2.10.2 Inconsistent BW lowering effect in humans

Mice have a high surface-to-volume ratio and a high metabolic rate [251] and BAT activity can account for up to 50% of the basal metabolic rate [270]. Therefore, the overall contribution of BAT thermogenesis to whole-body energy metabolism is substantial compared to obese humans who have less BAT [250]. Therefore, the mechanism by which FGF21 lowers BW is important to understand in order to predict human efficacy. There are conflicting data regarding the contribution of UCP-1 activity to FGF21-mediated BW loss [116, 117] but one study did show that no increase in EE is observed in FGF21 treated UCP-1 KO mice [116]. Furthermore, in mice the FGF21-induced EE can be blocked by a β3-AR inhibitor, the activation of which is required for activation of BAT [121]. Several sympathomimetic β3-AR receptor agonists, which had shown great BW lowering efficacy in rodents and monkeys, were terminated in phase 2 clinical trials due to lack of efficacy as reviewed by Peng et al. [271] and suggestions regarding the effects of FGF21 in humans need to be seen in that light, since it is difficult to translate effects of compounds that increase EE via activation of BAT (or inducing browning of white adipocytes) from mice to humans. The pronounced effect of FGF21 on EE observed in DIO mice and monkeys [6, 7] was not observed in chow-fed obese pigs [92](II) or in several of the NHP studies [9, 202] where FGF21 decreased BW by decreasing FI. Therefore, the composition of the diet (high fat low protein) may have facilitated non-shivering thermogenesis in HFD fed mice and monkeys. It is currently not known if the appetite-lowering effect of FGF21 is caused by differences in diet composition, food preference, compound or a dose-dependent effect. FGF21 analogues have been shown to lower BW in some studies [8, 9], while other clinical studies show no effect on BW [10, 194, 201]. It is moreover not known if the observed BW-lowering effect in humans [8, 9] is driven by a decrease in FI or an increase in EE. Furthermore, food preference, which has been described in mice and monkeys [59, 69], may also impact the effect of FGF21 on BW in humans, who have a free choice of food compared to animals in the pre-clinical species. Recently, it

has been shown that humans with the ability to increase plasma FGF21 acutely in response to a high calorie low protein diet are more susceptible to lose weight [272] suggesting that FGF21 influences BW in humans. However, as discussed, a SNP related to FGF21 loss of function is associated with a decrease in body-fat percentage but increased visceral obesity [214], and FGF21 treatment may therefore increase subcutaneous fat mass and BW in humans.

2.10.3 Strong lipid lowering effect in humans

Compared to the limited and varying effect of FGF21 analogues on BG and BW in humans, the lipid-lowering effect was more pronounced in humans compared to mice, pigs and monkeys [6, 16, 92](II and III). However, none of the applied pre-clinical models are great models for studying lipid-lowering effects. Part of the effect of FGF21 is to facilitate uptake of lipid into the adipose tissue and, notably, with respect to the discussion of the contribution of BAT in the BG and BW lowering effect, FGF21 has been shown to increase TG uptake into the BAT of obese mice [166]. Lowering of lipids may prevent development of CVD and rhFGF21 has been shown to decrease plaque formation in ApoE ko mice [175]. A potent and selective PPARα agonist pemafibrate [273] is currently in phase 3 clinical trial in patients with high plasma TG and as FGF21 is a down steam target of PPARα [55] these data are highly relevant in relation to the effect of FGF21 in humans. Pemafibrate was shown to reduce plasma TG levels by 50% in a phase 2 trial [273] and it will be interesting to see if pemafibrate can prevent CVD.

2.10.4 Other potential indications for FGF21 therapy

Other potential beneficial effects of FGF21 have been described in mice and humans. Pegbelfermin is currently in development for NASH and a beneficial effect on hepatic steatosis and fibrosis has been observed after 16 weeks of treatment [194]. The strong effect of FGF21 on hepatic steatosis significantly improves the NASH score independent of changes in BW [274]. Removal of hepatic fat will decrease the hepatic insult and lower inflammation and fibrosis, but direct effect of FGF21 on hepatic inflammation [275] and fibrosis [276] also have been described. Furthermore, FGF21 increases GSH biosynthesis, which protects cells against oxidative stress [277, 278]. Also, alcohol-induced [235] and acetaminophen-induced liver toxicity [279] may benefit from FGF21 therapy. Several pre-clinical studies indicated that FGF21 may improve cardio-health as reviewed by Cheng, et al. [280] as well as renal function [196]. In pre-clinical models, FGF21 has also been shown to protects towards cerulein-induced pancreatitis [281]. Furthermore, FGF21 has positive effects on cognitive impairment and multiple sclerosis in pre-clinical animal models [282, 283]. The many beneficial effects of FGF21 observed in pre-clinical species may be linked to FGF21's anti-oxidative effects [9, 284]. FGF21 may also prevent cancer by decreasing plasma IGF-1 and mTOR activity [285]. In general, plasma FGF21 is increased in several pathophysiological conditions and the increase in plasma FGF21 observed in obesity [286], heart failure [287-289], sepsis [290], vascular complications [291] and NASH [292] may play a protective role by altering substrate utilization and by reducing oxidative stress [293].

2.11 Side effects that may limit human therapy

Drug development involves a careful evaluation of benefits versus risks for the patient. Such an evaluation is performed early in development based on observations in pre-clinical animal models, which determine if it is safe to expose humans to a given molecule. The severity, the safety window (therapeutic index) and possibility for clinical monitoring are important factors to consider before conducting a clinical trial. Furthermore, it is also of importance to observe if a given side effect is reversible. In the FGF21 tg mice several adverse findings have increased the awareness of potential risks in exposing FGF21 to humans. However, no toxicological data of FGF21 or its analogues in rodents or higher species have been published.

2.11.1 Adverse effects on bone mineral density and female reproduction

In addition to the impaired linear growth [224] the FGF21 tg mice also have decreased bone mineral density (BMD) [294]. Furthermore, the female FGF21 tg mice are infertile and have delayed puberty development [183]. FGF21 treatment has been shown to decrease plasma IGF-1 [12] and increase plasma corticosterone [32], two factors known to affect BMD. As discussed previously, FGF21 tg mice have low plasma leptin levels [4, 32] and as leptin is important for normal hypothalamic functions like growth and reproduction [143], a decrease in plasma leptin may be responsible for the adverse findings in mice. During prolonged fasting [295, 296] and in women with anorexia nervosa (AN) [297] or lipodystrophy [298], plasma leptin is decreased and high energy-demanding processes such as female reproduction (hypothalamic amenorrhea) [299, 300], bone remodeling [297] and GH release [301] are suppressed while the HPA axis is activated [217, 302]. In line with this observation the estrus cycle in the FGF21 tg mice is rescued by high fat diet [303] and in humans the clinical observations caused by starvation or AN are also reversed if FI is increased. Interestingly, female mice fed a low protein diet also have decreased fertility and decreased BMD, but this is prevented by supplying an energy-rich diet [304, 305]. The high fat/high energy diet may therefore normalize plasma leptin in FGF21 treated mice and in PR mice. However, as discussed above no consistent decrease in leptin was no observed in monkeys treated with FGF21 [9, 193].

2.11.2 Biomarkers addressing safety

Several biomarkers addressing safety have been measured in pre-clinical species and in humans in response to FGF21 treatment and the data are summarized in Table 11. Measurement of biomarkers addressing female reproduction is complicated by the female sex-hormone cycles and in our non-rodent animal studies OVX animals have been used to ensure a constant intake of food [16, 92](II and III); therefore biomarkers addressing female fertility are not included in Table 11.

TABLE 11 BIOMARKERS ACROSS SPECIES ADDRESSING SAFETY (DATA FROM PUBLICATION II AND III)

Species	Mouse	Pig	Monkey	Human
GH	Increased [224]	Decreased	ND	ND
Total IGF-1	Decreased [224]	Decreased [92]	Decreased	Increased 40% at the highest dose [9, 201]
IGFBPs	Increased IGFPB2 [224]	ND	Increased IGFPB2 [16]	ND
Corticosterone/ Cortisol	Increased [130]	Unaltered	Unaltered	ND
BMD	Decreased [294] Unaltered [306]	No affected (14 weeks study) [92]	Not affected (12 weeks study) [16]	ND
Bone markers CTX Osteocalcin P1NP BSAP	1.5-fold increase in CTX increased 1.5-fold decrease in osteocalcin [294]	2-fold increase in CTX-1, no significant effect on osteocalcin [92]	2-fold increase in CTX-1, no significant effect on osteocalcin [16]	CTX dose dependently increased [201] [9] – while markers of bone formation were dose dependently decreased Minimal to no effect on bone markers [201]
BP	ND	ND	Not affected [201]	Systolic, diastolic BP, and pulse rate increased in a dose- and time-related manner 5 mmHg at highest dose) [201]

ND not determined

GH/IGF-1 axis: A major difference between FGF21 action in the pre-clinical species and human is the effect on total plasma IGF-1, which is decreased in pre-clinical species but increased in humans treated with PF-05231023 [9, 201]. GH is released from somatotrophic cells in the anterior pituitary

in a pulsatile circadian pattern with peaks levels at night [307]. GH increases the hepatic expression and secretion of IGF-1, which is the major contributor of circulating IGF-1. IGF-1 is also produced locally by other tissues and acts there in an auto/paracrine manner [308]. In the circulation IGF-1 is bound to the acid-labile sub-complex (ALS) and IGFBPs [309, 310] and the tertiary IGF-1, BPs and ALS complex stabilizes IGF-1 in plasma [311]. GH also increases the hepatic expression of IGFBP3 and ALS [312], while IGFBP1 and IGFBP2 are increased in the absence of GH [313]. FGF21 has in mice been described to cause GH resistance [224] as a decrease in plasma IGF-1 and an increase in plasma GH (one time point only) is observed. However, FGF21 treatment decreased peak GH release in obese mini-pigs and consequently also a decrease in plasma IGF-1 [92](II). Also, in lean rats FGF21 decreased GH peaks (unpublished data, B. Andersen). IGFBP2 has been shown to be increased in mice [224] and monkeys treated with FGF21 [16](III), indicating a reduction in GH activity but unfortunately no IGFBPs were measured in humans treated with FGF21 analogues. Notably, patients with high circulating GH are insulin resistant and have increased risk of developing diabetes [261]. Conversely, lack of GH, as observed in adult GH deficiency, has detrimental effect on health causing fragile bone, decrease muscle strength, increased visceral obesity and high prevalence of non-alcoholic fatty liver disease (NAFLD) [314]. Laron dwarfs with no circulating IGF-1 have increased longevity and reductions of age-related diseases like cancer and diabetes [315]. Interestingly, Laron dwarfs also have high circulating adiponectin [316]. Lack of GH has detrimental effect on lean mass and while mice [6] and monkeys [16](III) treated with FGF21 mainly lost fat mass, FGF21 treated pigs also lost lean mass [92](II). Thus, the right balance of the GH/IGF-1 axis is important to maintain good metabolic health [317] and it is of high importance to understand the effect of FGF21 on the GH/IGF-1 axis in humans. Interestingly, in humans, low protein intake is associated with a decrease in plasma IGF-1 and overall decreased mortality and lower cancer risk in middle aged individuals (50-65 years) while a high protein intake increased overall mortality and cancer risk [317]. Thus, both low protein intake and FGF21 therapy may optimize health span and longevity; however, GH is important for somatic growth and to maintain lean mass and the intended target population for FGF21 need to be carefully considered.

Glucocorticoids: Cortisol is a steroid hormone produced and released in the zona fasciculata of the adrenal cortex within the adrenal gland in response to ACTH. In the pituitary CRF triggers the release of ACTH. Cortisol is released in response to ACTH in a circadian manner and peaks in the morning [318]. Therefore, 24-hour plasma or urine profiles (24 hours collection) of cortisol are required to determine the effect of FGF21 on the HPA axis. FGF21 tg mice display increased CRF in the CNS and increased plasma corticosterone [130, 319] and the increase in CRF has been shown to increase EE. In humans the pre-partum elevation of cortisol is pivotal in the initiation of non-shivering BAT thermogenesis at birth and infants do as discussed above express high KLB in the CNS [98]. Moreover, individuals with active BAT also have higher plasma cortisol [320]. Therefore, it is important to highlight that pharmacological doses of FGF21 for more than 12 weeks did not alter the plasma cortisol in obese pigs or monkeys [16, 92](II and III) even though EE was increased in response to rhFGF21 in the high fat fed obese monkeys. However, an increase in postprandial plasma cortisol [321] may have contributed to the increase in EE in the monkeys, and this was unfortunately not measured. The effect of FGF21 on the HPA axis in humans is unknown and needs to be determined.

Bone markers: A potential safety concern related to FGF21 is the adverse effect on BMD. FGF21 tg mice have been reported to have decreased BMD and changes in bone markers [294]. Furthermore, a direct effect of FGF21 on chondrocytes has also been described [322]. PAPRγ agonists increase the risk for fracture after 2 years of treatment as reviewed by Vianna et al. [323], while a decrease in BMD is observed after 12 weeks of treatment with a low dose of dexamethasone [324]. It is, therefore, almost impossible to foresee if chronic FGF21 treatment will affect BMD in humans based

on pre-clinical studies. Compared to rosiglitazone, which in two weeks decreases BMD in mice, no decrease in BMD was observed in FGF21-treated mice [306]. Furthermore, obese mini-pigs and rhesus monkeys treated daily with FGF21 for 12-14 weeks showed no change in BMD [16, 92](II and III). Bone is constantly being remodeled also in adults. The bone tissue is resorbed (bone resorption) and concurrently rebuilt (bone formation). The bone turnover balance is important for repair of microfractures, but the bone mass does not change a lot in heathy male and premenopausal females. Bone markers are classified as markers of either marker of bone resorption or bone formation. Osteocalcin (also bone gamma-carboxy-glutamic acid-containing protein), N-terminal pro-peptide of type I procollagen (P1NP) and bone specific alkaline phosphatase (BSAP) are markers of bone formation, while Carboxy-terminal collagen crosslinks (CTX) is specific to bone resorption. FGF21 treatment increases plasma CTX (bone resorption) while bone formation markers are decreased [9, 16, 92](II and III) across species as seen in Table 11. This may however, be secondary to BW loss and interestingly, no consistent change in bone markers was observed in humans treated with PF-05231023 where no BW loss was observed [201]. Therefore, currently the effect of FGF21 treatment on bone health might be related to a decrease in BW, which is known to alter bone markers due to changes in weight bearing of the bone [325]. However, this if difficult to determine without a weight-matched control group.

Blood pressure: The FGF21 analogue PF-05102310 has been shown to increase BP in humans [201]. A similar effect has not been reported for other FGF21 analogues and likewise not reported in other clinical trials with PF-05102310 [202]. In mice and monkeys no adverse effect on BP has been observed [201] while an increased in BP is observed in FGF21-treated rats [201]. In contrast, FGF21 has been shown to protect towards ang II-induced hypertension in mice and to normalize systolic BP in hypertensive rats fed high-fructose in the drinking water [326, 327]. In addition, loss of FGF21 function in humans (GWAS) is associated with increased BP [214]. However, based on data from the PF-05102310 trial [201] and the ability of FGF21 to activate CRF and the SNS [121, 131], BP and heart rate should be closely monitored in the clinical setting. As FGF21 increases BAT activity in mice and a high bolus injection of FGF21 has been shown to increase body temperature (BT) [6, 107] effect on BT also need to be included in human trials. However, mice have much more BAT compared to humans and it is therefore unlikely that FGF21 will change BT in humans.

2.11.3 Adverse effects observed in human trials

None of the human trials have determined the effect of FGF21 on BMD, and long-term studies are required to address this. Additionally, female fertility must be monitored if women of child-bearing potential are included in the studies. The most common side effects reported at increasing doses in the clinical setting are gastrointestinal (GI) side effects such as diarrhea, nausea, increased appetite and vomiting [9, 201, 204]. The GI-related side effects observed in humans have not been observed in pre-clinical species but could be related to lack of appetite observed in several of the NHP studies [9, 68] as well as in the obese mini-pigs [92](II). The explanation for the GI side effects is unclear, but FGF21 is an exocrine pancreas secretagogue in mice [328] and FGF21 may affect bile acid metabolism [87] and changes in food absorption and GI tract mobility may be anticipated.

In summary, several of the observed side effects in mice are likely linked to low plasma leptin and lack of energy and seem to be reversible by addition of a high fat diet [303, 305]. Furthermore, no decrease in BMD was observed in obese pigs [92](II) or NHP [16](III) treated with FGF21 despite a significant weight loss while bone markers were altered. Changes in bone markers have also been reported in humans but only in a study where a significant decrease in BW loss was observed [9]. The side effects observed in mice caused by a potential lack of energy (low leptin) is not likely to be

observed in obese humans but clinical monitoring of BP and HR as well as biomarkers addressing potential negative effect on bone health should of course be included in future clinical trials.

2.12 Summary pharmacology

Since the first pharmacological effect of FGF21 was reported in 2005 [4] the knowledge about FGF21 has increased significantly. Several FGF21 analogues have been tested in clinical trials confirming that FGF21 has metabolic activities in humans [8-10, 194]. While a pronounced plasma lipid-lowering effect of FGF21 analogues is observed in humans, the BG and BW lowering effects vary across studies. In humans FGF21 analogues increase insulin sensitivity [8, 10] indicating that FGF21 may increase non-insulin dependent glucose uptake. Furthermore, FGF21 facilitates lipid uptake into the adipose tissue and if the substrates are not utilized as energy, the extra energy is stored as lipids with a potential of increasing fat mass [305]. Notably in FGF21-treated pigs, a significant BW loss did not improve body composition [92](II) which may however also be related to a decrease in IGF-1 [92](II). A BW lowering effect of FGF21 is observed across pre-clinical species mediated by either increases in EE [6, 7, 16](III) or a decrease in FI [92, 202](II). HFD seems to induce FGF21-mediated increases in EE in mice and monkeys [6, 7, 16](III) while a decrease in FI is observed in response to FGF21 in chow-fed monkey and pigs [92, 202](II). Furthermore, pre-clinical data [59, 68, 69] and GWAS results suggest that FGF21 affects food preferences [241] and this is not adequately reflected in the pre-clinical studies with fixed diets. Therefore, these findings support the hypothesis that the effect of EE and FI of FGF21 across studies and species may be influenced by the macronutrient content of the diet. The effect of diet composition therefore needs to be taken into consideration when interpreting observations in pre-clinical FGF21 studies. Therefore, additional studies are required to understand if the ability of FGF21 to increase EE or decrease FI is affected by the dietary composition in pre-clinical studies. Also, doses need to be taken into consideration as higher doses of native FGF21 trended to suppress FI in high fat-fed monkeys [16](III). Furthermore, future clinical studies need to address the effect of FGF21 on FI, EE, food preferences and as well as BW. The pronounced lipid-lowering effect of FGF21 analogues observed in humans as well as the increases in anti-oxidative stress markers (SOD and GSH) observed in mice [175] may protect towards development of CVD. Furthermore, the significant decrease in hepatic steatosis and fibrosis in patients with NASH after 16 weeks of treatment with pegbelfermin [194] support further clinical evaluation of FGF21 for the treatment of NASH where no therapy is currently available. The major risks associated with FGF21 therapy are currently effects on BMD and hemodynamic changes, and other side effects may potentially appear in longer clinical studies.

3 PART II: The physiological role of FGF21

3.1 Introduction

The beneficial effect of FGF21 on BG and BW in mice and NHP significantly moved the field towards identification of optimized FGF21 analogues suited for human therapy. However, the physiological role of FGF21 remained poorly understood. FGF21 was initially suggested to play a major role in the adaptive response to fasting contrasting the pharmacological effect of FGF21 on EE and insulin sensitivity. The second part of the thesis concentrates on the hypothesis that FGF21 activity is down-regulated in response to fasting in humans. This will allow flux of FFA to non-adipose tissue, decrease EE and insulin sensitivity. Conversely, plasma FGF21 and activity is increased in response to high glucose and protein deficiency. FGF21 is involved in the metabolic adaptation required to adjust for low protein intake which may involve changes in the GH/IGF-1 axis.

3.2 Regulation of FGF21 and FGF21 receptor complex

3.2.1 FGF21 expression

Analysis of DNA and coding sequences of *Fgf21/FGF21* genes reveals that the sequences have been maintained by natural selection, and that the structures of the Fgf21 genes are highly conserved in vertebrates and mammals [329]. In mammals, the *Fgf21/FGF21* gene is highly expressed in liver and pancreas while a lower expression is observed in adipose tissue and skeletal muscle [21, 50-54](VII). As discussed previously, the three major regulators of hepatic FGF21 expression are FFA [12, 15, 55], glucose [58] and lack of amino acids [60]. FGF21 is also expressed in WAT [21, 99](IV) as well as in BAT [147, 330] and the expression of FGF21 is increased in response to adrenergic receptor activation [331], insulin [53, 54](VII) and PPARγ agonism [51]. FGF21 expression has also been detected in skeletal muscle [99, 332](IV) in response to insulin treatment [53, 54, 333](VII) and exercise [334]. FGF21 expression has been observed in the CNS of rats [181] as well as humans [182] and FGF21 is present in human cerebrospinal fluid [178, 179]. In mice the circulating FGF21 has been shown to be derived from the liver [114] and in humans with NAFLD plasma FGF21 also closely correlates with hepatic mRNA expression of *FGF21* [335]. However, FGF21 is also released from other tissues and plasma FGF21 derived from skeletal muscle has been suggested as a biomarker of mitochondrial diseases [336]. FGF21 expression is in general increased in response to various stress responses in liver, heart, kidneys and pancreas as reviewed by Gomez-Samano, et al. [284].

3.2.2 The FGF21 receptor complex

FGF21 only binds to FGFRs in the presence of KLB and the expression of KLB is essential for FGF21 activity. The adipose tissue [21, 54, 57, 72, 91, 99, 103](IV and V) and specific regions of the CNS [32, 97] express KLB and the FGFR1c or FGFR3c. As KLB and the short isoforms of FGFR1 and FGFR3 are essential for metabolic activity of FGF21 [27] regulation and expression of these receptors influence FGF21 activity [54, 88]. Limited information has been published on the regulation of the FGF21 receptor complex. In the adipose tissue, low grade inflammation decreases KLB expression [337], while GLP-1 receptor agonism [338], exercise [339], cold exposure [340] and PPARγ agonism [339, 341] increase the expression of KLB in adipose tissue.

Therefore, FGF21 released from the liver enters circulation and act on target cells in the CNS and the adipose tissue while FGF21 released form the adipocytes functions in an autocrine/paracrine manner. Oppositely, FGF21 released from the skeletal muscle in response to insulin is believed to work as an endocrine (myokine) agent as the skeletal muscles do not express KLB [21, 54, 99](IV). A simplified figure of FGF21 expression and regulation in liver, adipose tissue and skeletal muscle is shown in Figure 3.

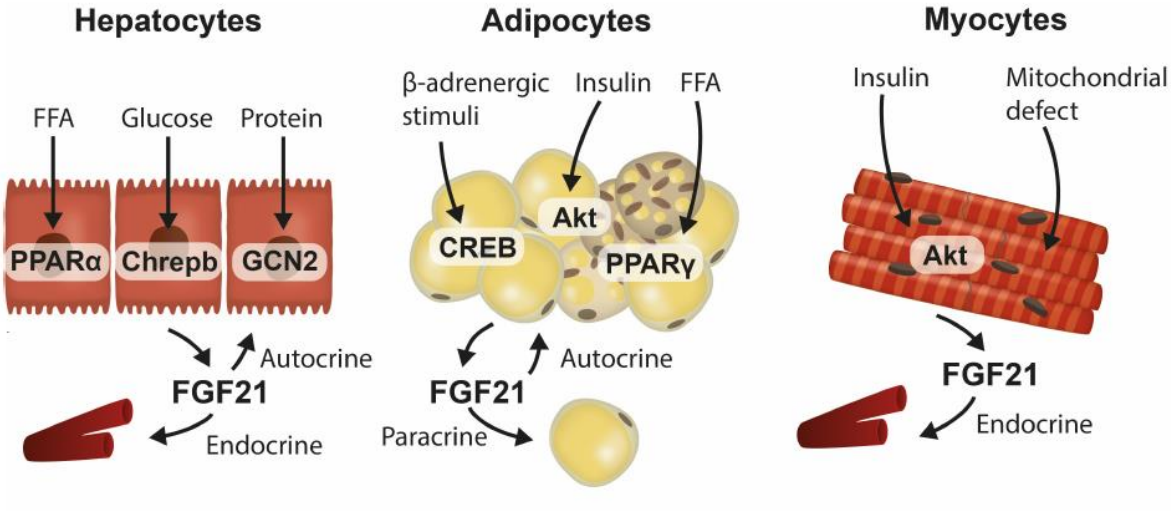

FIGURE 3 FGF21 EXPRESSION IN HEPATOCYTES, ADIPOCYTES AND IN SKELETAL MUSCLE

FGF21 is expressed in hepatocytes in response to FFA, glucose and lack of amino acids. In adipocytes FGF21 is expressed in response to β-adrenergic stimuli, insulin and FFA, while FGF21 expression in skeletal muscles occurs in response to insulin and in response to mitochondrial defects. Plasma FGF21 is mainly liver derived. As the FGF21 receptor complex is not expressed in skeletal muscles, FGF21 released from muscle work in an endocrine fashion. The circulating FGF21 is mainly liver derived but FGF21 released from hepatocyte can also activate signaling pathways in hepatocytes. FGF21 released from adipocytes works in autocrine manner.

3.2.3 Measuring Plasma FGF21

In mice [148], monkeys [99](IV) and humans [53, 57, 247, 342](V, VI, VII, and VIII), the basal plasma concentration of FGF21 is 100-200 pg/ml. Gender differences have been described after adjustment for age and BMI [343]. Large interpatient variations have also been observed in several studies [247, 344, 345](VIII). The reason for the large interpatient variation in some studies is currently not understood, but FGF21 may bind to plasma proteins (e.g. FGF binding proteins), which interfere with binding of antibodies applied in some of the enzyme-linked immunosorbent assays (ELISA). Recently, FGF binding protein 3 (FGFBP3) was found to change the activity of FGF21 *in vitro* [346], indicating that FGF21 binding proteins may be present in plasma. Circulating FGF21 is proteolytically cleaved at its N-terminus by DPP-4 [347], while the C-terminus is cleaved between Pro-171 and Ser-172 by fibroblast activating protein (FAP) [347, 348]. Deletion of the N- and or C-terminal portions of FGF21 leads to an inactive protein [37]. Therefore, specific antibodies targeting the N- and C-terminal of FGF21, have recently been developed to distinguish between total and active FGF21 [248, 349]. An increase in plasma FAP has been observed in various diseases like chronic liver disease [350] which may therefore decrease active FGF21 in plasma. Most literature studies have measured total plasma FGF21, which may overestimate active FGF21 protein. In the future, applications of specific antibodies targeting the active form of FGF21 may reveal new connections and increase our understanding of FGF21 biology. In addition to expression and secretion, plasma FGF21 may also be regulated by elimination and plasma FGF21 is increased in patients with renal impairment (up to 4000 pg/ml) potentially due to decreased clearance [351-353]. Moreover, as FGFR are tyrosine kinases, FGF21 clearance by internalization may also take place [354]. Plasma FGF21 displays a circadian variation [342](VI) so time of sampling is important and needs to be taken into consideration. In the following sections plasma FGF21 is "total FGF21" and not FGF21 (1-181) unless stated. However, active FGF21 (1-181) seems to follow similar pattern as total FGF21 [349] even though this may very between different physiological and pathophysiological stages as just discussed.

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3.3 FGF21 and Long-Term Fasting

3.3.1 Metabolic adaptations to fasting

The ability to adapt to long term fasting/starvation (chronic undernutrition) has been of utmost importance for our survival in times of famine. The adaption to fasting in mammals involves a decrease in plasma insulin and in glucose oxidation. Initially, the stored glycogen in the liver and skeletal muscle is used for energy and later lipids stored in the WAT become the major energy source by releasing glycerol and FFA for glucose and ketone bodies (acetoacetic and β-hydroxybutyric acids) production. Also, skeletal muscles supply the liver with gluconeogenic amino acids and ketogenic amino acids during prolonged starvation. In response to fasting, glucagon is released from the pancreatic α-cells following a decrease in BG [355] and insulin [356]. Increases in plasma glucagon increase hepatic glucose [357] and ketone body production, which depends on elevation in carnitine content [358]. Low BG also increases plasma epinephrine levels which may contribute to hepatic glucose production [359]. The decrease in BG during fasting also increases ghrelin release from the stomach [360] and acts as a GH secretagogue [361] while also low plasma IGF-1 also stimulates GH release [362]. The increase in plasma GH in response to fasting is important to induce lipolysis [363, 364] and high GH also facilitates the swift from carbohydrate to fat utilization (Randle cycle [365]) promoting fatty acid oxidation [366, 367], keeping protein oxidation to a minimum, as reviewed by Møller and Jørgensen [368].

3.3.2 Fasting and FGF21 in mice

Hepatic *Fgf21* mRNA expression and plasma FGF21 levels increase 2- to 7-fold (from 260 pg/ml to 1700 pg/ml B. Andersen, unpublished observation) in response to fasting in lean mice [13, 15, 272]. Increases in plasma FFA induce FGF21 expression by activation of transcription factor PPARα [12] and no increase in plasma FGF21 is observed in the PPARα KO mice in response to fasting [13]. Mice lacking FGF21 have been reported to have an impaired response to fasting (gluconeogenesis and ketogenesis) [168] but these initial findings were not confirmed by others [62, 233]. However, lack of FGF21 causes hepatic lipid accumulation [233] and the PPARα KO mice display a similar phenotype and develop fatty liver in response to fasting which may reflect an impairment in hepatic fatty acid oxidation [369] However, as FGF21 inhibits insulin secretion, lack of PPARα and FGF21 potentially fails to suppress insulin secretion [89], which will prevent fatty acid oxidation [369]. Initially, FGF21 was shown to increase lipolysis in 3T3-L1 adipocytes [12] and FGF21 was assigned as “the missing link in the biology of fasting” [11]. The effect of FGF21 on lipolysis has, however, been quite controversial and the initial finding was not confirmed by other groups, who have shown that FGF21 inhibits lipolysis and stimulated lipogenesis in 3T3-L1 adipocytes [94]. Furthermore, treatment with rhFGF21 acutely inhibits lipolysis [128] by reducing phosphorylated hormone sensitive lipase (HSL) [135] and adipose triglyceride lipase (AGTL) expression [63]. However, the decrease in FFA may also be secondary to increase utilization in, for example, the BAT [153] and additional measurements of plasma glycerol would help reveal how FGF21 lowers plasma FFA. Fasting also decreases insulin sensitivity [370] and EE [15] in contrast to FGF21 treatment, which increases insulin sensitivity and EE [6, 7, 16](III). Thus, it seems controversial that the physiological role of FGF21 would be completely opposite to what had been observed in response to FGF21 treatment.

3.3.3 Regulation of plasma FGF21 in response to fasting in lean subjects

The increase in plasma FGF21 in lean mice in response to fasting [13, 272] initiated several studies in humans. Mice have a large surface-to-BW ratio and a high metabolic rate that force them to consume 10% of their own BW every day to prevent BW loss. Therefore, an overnight fast in mice

may equal 7-10 days of fasting in humans. In 2008, 7 days of fasting in 5 lean subjects with rheumatic arthritis (RA), surprisingly, only increased plasma FGF21 1.7-fold (from 913 pg/ml to 1592 pg/ml) [344]. The initial plasma FGF21 level of > 900 pg/ml is rather high and may be associated with RA and increased inflammation in these patients [290, 371]. Furthermore, the samples in this study [344] originated from a previous study where 14 patients were included [372]. Therefore, fasting may actually have decreased plasma FGF21 in some of the 14 subjects, as the inflammatory state in the 14 patients was shown to decrease with fasting [372].

No change in plasma FGF21 was found in response to 48 hours [344] or 72 hours of fasting [373] in humans. This is in agreement with our two studies, where no significant change in plasma FGF21 was observed in healthy female volunteers fasted for 72 hours [342] (VI) or in healthy males fasted for 60 hours [57](V). However, in 2015, a study showed that 10 days of fasting increases plasma FGF21 3-4-fold in healthy subjects [15]. In humans, plasma FFA has been positively correlated to plasma FGF21 [374, 375] indicating that hepatic FGF21 is increased in response to PPARα in humans. However, no increase in plasma FGF21 was observed in response to more than 24 hours of fasting plasma [344, 373] despite a 2-fold increase in plasma FFA [342](VI). Conversely, in mice plasma FFA only increased 30% in response to 24 hours of fasting [13] while plasma FGF21 is increased several-fold, potentially illustrating a large increase in FFA utilization in the fasted mice. Despite no increase in plasma FGF21, ketone bodies are already increased after 24 hours of fasting in humans [15, 342, 344](VI) and furthermore no hypoglycemic events occurred in response to fasting (3-10 days)[15, 57](V). This suggest that FGF21 is not required for ketogenesis or to maintain normal glycemia in humans, in agreement with studies in mice [62, 233].

Interestingly, during the 10 days of fasting the increase in plasma FGF21 occurred very late (from day 9 to day 10) [15], and it is unclear which factors are involved in the late increase in plasma FGF21 [15, 344]. Cortisol and GH, which have been shown to stimulate FGF21 [56, 319] and lipolysis [376, 377], are already increased after a few days of fasting in humans [342, 355, 378, 379](VI). Moreover, no increase in plasma FGF21 is seen in patients with Cushing [380] or in response to GH treatment in healthy individuals [381], excluding cortisol and GH as direct or indirect (via lipolysis) drivers for the late increase in plasma FGF21. However, exogenous glucagon increases plasma FGF21 1.5-fold [382] but glucagon in already increased after 1-2 days of fasting [342](VI) and does not further increase in response to fasting [15]. As lipolysis is significantly increased in response to fasting, hepatic lipid accumulation takes place and in healthy humans fasting increases hepatic TG 1.5-fold after 48 hours [383]. This does, however, not increase plasma FGF21 in any of the published studies [57, 342, 344, 373](V and VI) despite several studies shown positive correlation between hepatic steatosis and plasma FGF21 [292, 384]. As lack of protein is a major regulator of plasma FGF21 [60], changes in plasma amino acids in response to fasting may affect FGF21 expression. Interestingly, after an initial increase in plasma branched chain amino acids (BCAA) (from day 1 to day 7) in response to fasting in healthy subjects, plasma BCAA starts to decline [15, 385]. Therefore, the late increases in plasma FGF21 may be associated with an eventual lack of amino acids and future studies are suggested to correlate changes in amino acids and plasma FGF21 in response to fasting.

In conclusion, an increase in plasma FGF21 is observed in mice (24 hours) and human (10 days) in response to fasting. The high metabolic rate in mice requires a rapid adaptation to fasting and may enhance the signal in the mice. Furthermore, mice express 10-20-fold higher levels of hepatic PPARα compared to humans [386]. Additionally, an overnight fast in mice may be enough to induce protein deficiency and the increase in plasma FGF21 may be stimulated by induction of PPARα [60] and GCN2 [60]. In humans, the late increase in plasma FGF21 may also be related to a decrease in plasma amino acids [15, 385] inducing hepatic FGF21 via GCN2 [60].

3.3.4 Regulation of plasma FGF21 in response to fasting in obese subjects

Plasma FGF21 is positively correlated to BMI and insulin resistance in humans [286, 373, 387-389]. Liver fat is also positively correlated to plasma FGF21 [292, 384], as described above, and obese and diabetic subjects commonly display hepatic steatosis [390] as well as increased plasma FGF21 as reviewed by Keuper, et al. [185]. Therefore, we found it interesting to observe that 60 hours of fasting decreased plasma FGF21 (from 300 to 100 pg/ml) in obese subjects [57](V) as seen in Figure 4A. On average, no change in plasma FGF21 was observed in the lean subjects as discussed above, but plasma FGF21 increased after 12 hours in one subject as seen in Figure 4B. The subjects did not lose BW in response to the short fast, but the fast may potentially have decreased liver fat [391] and improved insulin sensitivity in the obese subjects [387] causing a normalizing of plasma FGF21. Interestingly, no significant correlation between plasma FFA and plasma FGF21 was found in lean or obese subjects [57](V), potentially due to the limited number of subjects. However, before initiation of the fast, plasma FFA and FGF21 trended toward a positive correlation (not significant) [57](V). At the end of the fast a negative correlation between plasma FGF21 and FFA was observed in the obese subjects [57](V), indicating a reverse relationship between FFA and plasma FGF21. To investigate a direct correlation between FFA and plasma FGF21 frequent plasma sampling is required [392]. It is furthermore, not known if plasma FGF21 will start to increase after 10 days of fasting in obese subjects as observed in lean subjects [15]. Because insulin resistance has been closely associated to plasma BCAA [265], changes in plasma amino acids, FFA and FGF21 in obese insulin-resistant subjects in response to fasting would be highly relevant to examine.

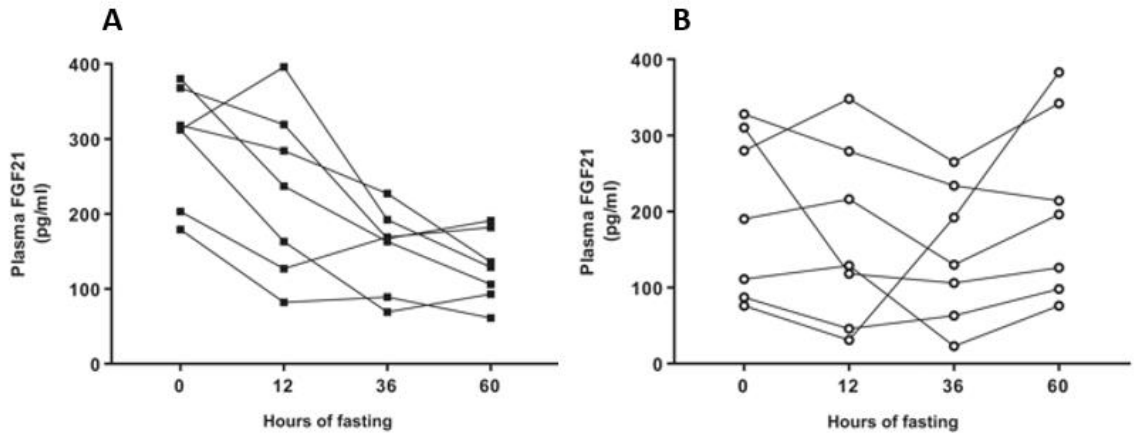

FIGURE 4 EFFECT OF 60 HOURS OF FASTING IN OBESE (A) AND LEAN SUBJECTS (B). (DATA FROM PUBLICATION V)

Individual plasma levels of FGF21 during fasting in obese (A) and lean (B) subjects.

3.3.5 Plasma FGF21 displays a circadian rhythm despite short term fasting

Many aspects of physiology are influenced by the circadian clock, including sleep-wake cycles, cardiovascular activity, endocrine system, body temperature (BT), etc. [393, 394]. During our investigation of the regulation of plasma FGF21 in response to fasting, we made the exciting observation that plasma FGF21 undergoes circadian variation as seen in Figure 5A [342](VI). Plasma FGF21 peaked around 2:30 in the morning and reached its lowest level six hours later (at 8.30 am) [342](VI). FGF21 in mice also displays a circadian regulation [395] and it has been shown to be regulated by several transcription factors influenced by the circulation clock, for example, Rev-Erbα

[395, 396], RAR-related orphan receptor RORα [397] and PPPAα [397-399]. FGF21 is described to be a second order clock-controlled gene product as reviewed by Erickson and Moreau. [400]. The circadian regulation of plasma FGF21 has been associated with diurnal changes in plasma FFA [392], which peak 1-2 hours before plasma FGF21 [342](VI). In the adipose tissue the lipolytic pathway is also under circadian control [401] and lipolysis is increased during sleep in mice [402] and humans [403]. In agreement with fasting, plasma FFA increased steadily in humans, however, a slight but significant decrease in FFA was observed at the time points where plasma FGF21 peaked [342](VI) as seen in Figure 5B. This is in agreement with FGF21's inhibitory effect on lipolysis [128]. It is therefore of interest to observe if the circadian regulation of plasma FFA is altered in FGF21 KO mice [62] or in humans with potential lack of FGF21 function [214]. The circadian rhythm of plasma FGF21 persisted during the 72 hours fast, but after 2 days of fasting the circadian rhythm of plasma FGF21 was less pronounced as seen in Figure 5A [342](VI). This observation is in agreement with studies in mice showing that the circadian regulation of FGF21 is impaired by caloric restriction [404] and by high fat feeding [405]. In humans, both obesity [392] and mild cold exposure [406], which increase circulating FGF21, abolish the diurnal regulation of plasma FGF21. It is currently not known how prolonged fasting/starvation (>10 days), which is known to disturb the circadian rhythm [407], affects the circadian regulation of plasma FGF21 in healthy and in obese subjects. Furthermore, it is unlikely that the circadian rhythm of plasma FGF21 persist during PR which significantly increases plasma FGF21 [60].

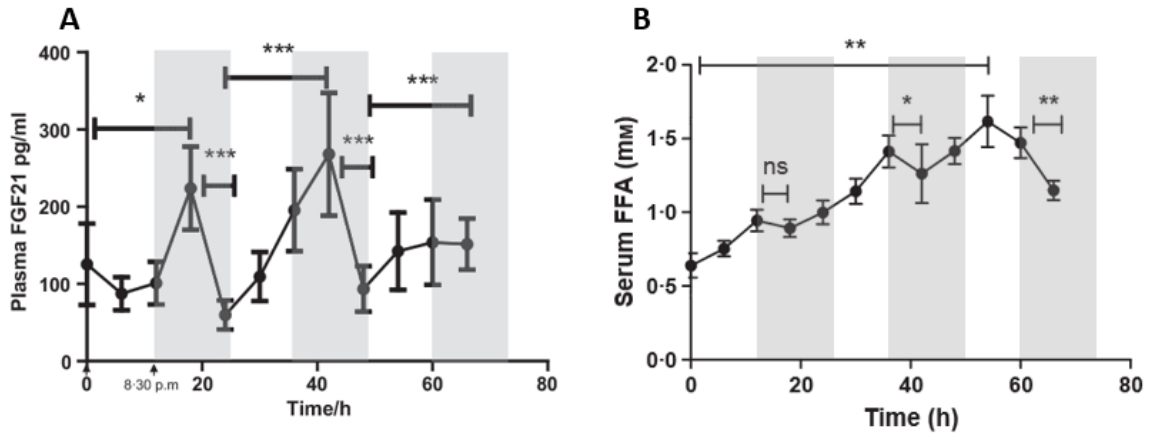

FIGURE 5 CIRCADIAN REGULATION OF PLASMA FGF21 AND FFA (DATA FROM PUBLICATION VI).

*Plasma FGF21 profile during 66 h of fasting in 14 healthy female subjects (A). The grey area indicates night-time starting at 8.30 p.m. and ending at 8.30 a.m. Serum free fatty acids profile (B) Values are mean ± SEM. Statistical significant differences between selected time points *P < 0.05, **P < 0.01and ***P < 0.001.*

3.3.6 Regulation of FGF21 receptor complex during fasting in lean and obese subjects

FGF21 treatment inhibits lipolysis and decreases plasma FFA [128]. Therefore, it would be more logical if fasting decreased plasma FGF21, allowing flow of FFA from the adipose tissue to non-adipose tissues. Therefore, it is rather interesting that three days of fasting decreased the mRNA expression of *KLB* and *FGFR1c* in WAT in both lean and obese subjects as seen in Figure 6 [57](V). A decrease in *KLB* and *FGFR1* mRNA expression in WAT was also observed after 10 days of fasting in the study by Fazeli et al. [15]. Furthermore, EE, which is increased in response to FGF21 treatment in

mice [6] and NHP [16](III), was decreased in response to 10 days of fasting [15, 408]. Additionally, plasma adiponectin, which is increased by FGF21 treatment in mice [108], NHP [16](III) and humans [8] is decreased after ten days of fasting [15], supporting a decrease in FGF21 activity in response to fasting. Thus, long term fasting/starvation, which decreases EE [15] and induces lipolysis [57, 342](V and VI), seems to be associated with a decrease in FGF21 activity in humans. A prerequisite for this proposal is that down-regulation of KLB and FGFR1c mRNA in the adipose tissue decreases FGF21 activity. The regulation of the KLB and FGFR is the CNS in response to fasting in humans is not known. It is furthermore not known if *Klb* and *FgfR1c* gene expression in the WAT are regulated in response to fasting in mice.

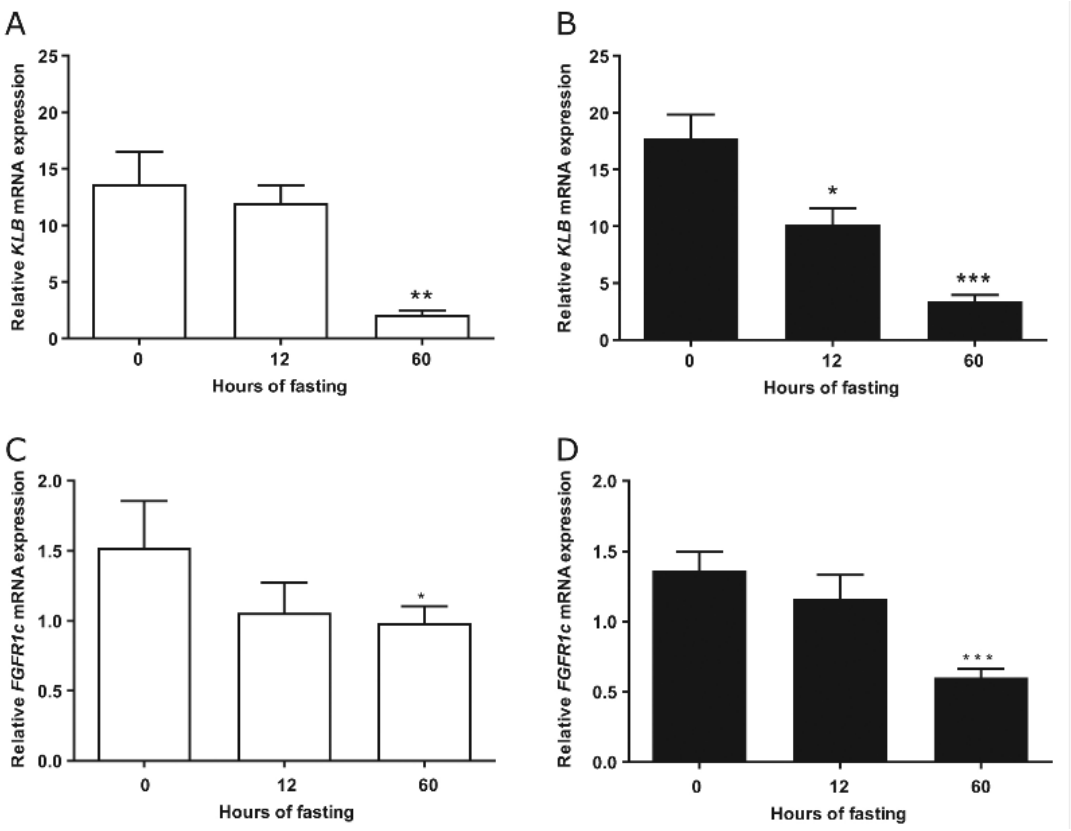

FIGURE 6 REGULATION OF KLB IN WAT DURING FASTING IN LEAN AND OBESE SUBJECTS (DATA FROM PUBLICATION V)

Expression of KLB and FGFR1c is downregulated in adipose tissue upon fasting. mRNA expression of KLB in lean (A) and obese (B) subjects during fasting. Changes in FGFR1c in lean (C) and obese (D) subjects during fasting. Results are mean ± s.e.m., analyzed by repeated-measures analysis, n = 5–7, denotes statistically significant differences at *P < 0.05, **P < 0.01, ***P < 0.005.*

3.4 FGF21 resistance in obesity

Plasma FGF21 is increased in obesity [88, 99, 286, 373](IV) while the expression of mRNA of *KLB* is decreased in adipose tissue from obese mice [88, 339], rats [327], monkeys [99](IV) and humans [54, 409]. Therefore, the ability of FGF21 to suppress lipolysis, induce glucose uptake and increase EE may be impaired in the obese state. Interestingly, *KLB* mRNA expression is decreased in WAT in high fat-fed monkeys which gain most BW on the high fat diet [99](IV). Monkeys which were resistant to BW gain had higher *KLB* mRNA expression in the WAT compared to monkeys sensitive to BW gain [99](IV). Therefore, the monkeys that were able to maintain expression of the FGF21 co-receptor in WAT in response to HFD were able to resist BW gain and to prevent development of dyslipidemia and hyperglycemia [16](III). However, inconsistent results on the expression level of KLB and FGFR1c

in WAT in human obesity exists. An increase in *KLB* and *FGFR1c* mRNA expression in visceral and SQ WAT has been observed in obese compared to lean subjects [65, 391], while other studies have shown a clear decrease in *KLB* mRNA in WAT of obese subjects [409]. The discrepancies may be related to degree of insulin resistance, which is related to more than just BMI (e.g., waist-to-hip circumference), across the different cohorts. Plasma FGF21 has been shown to correlate with the expression of thermogenic genes in subcutaneous WAT in humans; however, the correlation is lost in obese patients indicating FGF21 resistance [410]. In agreement with a potential decrease in FGF21 activity in obese subjects postprandial activation of the SNS is blunted in obesity [252]. The induction of diet-induced thermogenesis can, however, be restored by BW loss [411] and in mice [412] and humans [413] a very low-calorie diet (VLCD) decreases plasma FGF21. It is unknown if a VLCD restores FGF21 sensitivity, but BW loss restores postprandial increases in plasma cortisol [414], which may indicate an increase in postprandial FGF21 activity. In mice, exercise has been shown to restore KLB expression in the adipose tissue and the beneficial effect of exercise has been shown to depend on the upregulation of *Klb* mRNA in WAT [339]. Therefore, in contrast to fasting which decreases FGF21 activity, BW loss induced by dietary intervention and exercise seems to induce FGF21 sensitivity.

3.4.1 Regulation of FGF21 in response to Bariatric Surgery

Roux-en-Y gastric bypass (RYGB) surgery does not only lower BW but also reverses T2D [415]. After the surgery the food bypasses most of the stomach and increases the release of gut hormones like GLP-1 and PYY [416]. Changes in plasma FGF21 have been investigated in response to bariatric surgery with differing results. One study found no changes in plasma FGF21 one year after surgery [417] while other studies found an increase in plasma FGF21 three weeks and three months after surgical intervention [413, 418, 419]. Yet another study showed that the initial increase in plasma FGF21 was reversed to pre-surgery levels three, six and 12 months after the surgery [419]. Therefore, the change in plasma FGF21 in response to bariatric surgery is dependent on when after the surgery plasma FGF21 is measured. We observed no significant changes in plasma FGF21 1-2 weeks after Roux-en-Y bypass, but as morning plasma FGF21 levels were measured on three different occasions in each individual, before and after surgery, we were able to observe a significant increase in plasma FGF21 in response to surgery in three patients out of eight patients [247](VIII). The significant increase in plasma FGF21 in these three patients did, however, not correlate with additional loss of BW or other metabolic changes. Interestingly, Fjeldborg, et al. found a significant reduction in plasma FGF21 12 months after surgery in the group of obese patients who initially had the highest plasma FGF21 [391]. Furthermore, an increase in *KLB* and *FGFR1*c mRNA expression in subcutaneous fat was observed [391]. This increase in KLB expression may be secondary to the increase in GLP-1 release [416], which has been shown to increase after surgery [416] and to increase KLB expression in adipose tissue in mice [338]. Other mechanisms such as a decrease in inflammation in the WAT [420] may have contributed to an increase in FGF21 receptor complex expression [337]. The early increase in plasma FGF21 observed in response to bariatric surgery by some investigators [413, 418, 419] may also be caused by surgical stress [421] and the late decrease in plasma FGF21 may potentially be due to metabolic improvement e.g., increased insulin sensitivity. In obese mice, plasma FGF21 is decreased in response to bariatric surgery, but FGF21 plasma levels were higher after surgery compared to weight-matched controls, where BW loss was induced by low calorie diet [412]. However, FGF21 is not required for the metabolic benefit of bariatric surgery in obese mice, as BW, FI and improvement in glycemic control after surgery are similar between FGF21 KO and WT mice [412]. Therefore, it is unlikely that the reported increases in plasma FGF21 contribute to the beneficial effects of surgery [412]. Yet, bariatric surgery may increase FGF21 sensitivity in humans [391] as observed in mice [422].

3.5 FGF21 and nutrients

Hepatic FGF21 is regulated by PPARα, ChREBP, and GCN2 activated by FFA, glucose and lack of amino-acids, respectively. Therefore, changes in dietary macronutrients are expected to regulate hepatic FGF21 expression.

3.5.1 Regulation of FGF21 in response to glucose

Mice lacking ChREBP fail to increase plasma FGF21 in response to high glucose [58] and these mice develop hepatic steatosis, secondary to increases in lipolysis and plasma FFA [423]. In humans, plasma FGF21 is increased in response to an OGTT [424] and the response is enhanced after RYGB surgery as seen in Figure 7A and B [247, 425](VIII). The increase in plasma FGF21 after surgery has been suggested to be caused by the rapid delivery of glucose into the small intestine and the liver [425]. Interestingly, as seen in Figure 7D, no increase in plasma FGF21 was observed, either before or after surgery, if protein was given simultaneously (Nutridrink). This was observed despite an almost similar increase in plasma glucose and insulin [247](VIII). Therefore, lack of protein and high glucose acutely increases plasma FGF21, in agreement with the sympatho-excitatory effect of carbohydrate [426]. Therefore, bariatric surgery, which increases plasma FGF21 in response to an OGTT, may potentially also re-establish the sympatho-excitatory effect of carbohydrates by increasing plasma FGF21. Further, the increase in plasma FGF21 in response to an OGTT may also facilitate glucose uptake into adipocytes [4] and decrease further intake of glucose [59].

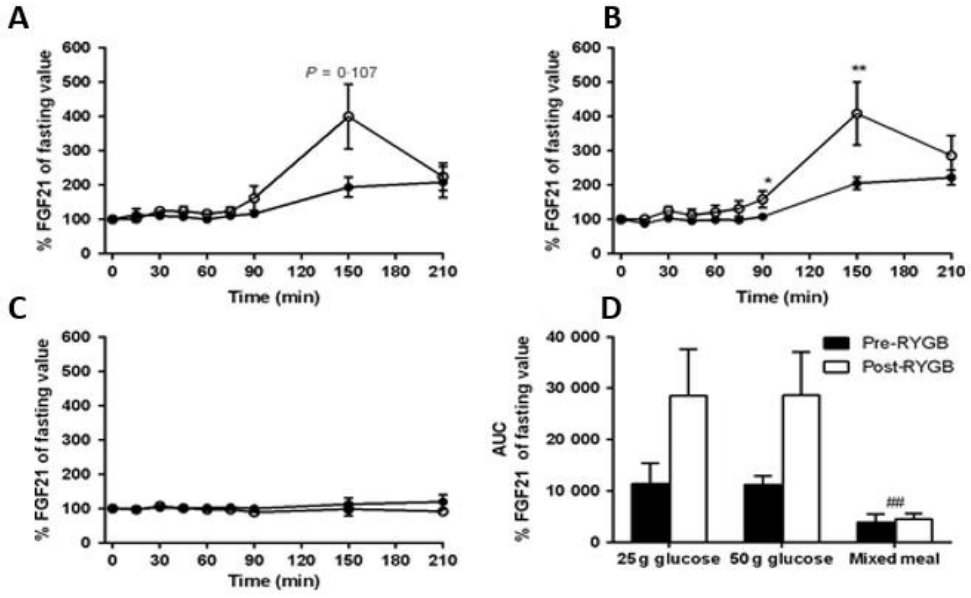

FIGURE 7 PLASMA FGF21 IN RESPONSE TO GLUCOSE AND A MIXED MEAL **BEFORE AND AFTER BARIATRIC SURGERY** (DATA FROM PUBLICATION VIII)

*Normalized postprandial plasma FGF21 before and after surgery. Plasma FGF21 in response to 25-g glucose (A) and 50-g glucose (B) and in response to a mixed meal (C). Closed symbols show FGF21 plasma levels before surgery, while open symbols represent FGF21 levels after surgery. Areas under the curve (AUCs) in response to the nutritional challenges (D). Closed bars show AUCs before surgery and open bars after surgery. Asterisks denotes significant differences (*P < 0·05, **P < 0·01) between the postprandial FGF21 responses before and after surgery.*

Increases in plasma FGF21 have also be shown to be induced by insulin postprandially [349]. The insulin-mediated postprandial increase in plasma FGF21 has been suggested to increase insulin

sensitivity [349]. Furthermore, a hyper-insulinemic euglycemic clamp also increased plasma FGF21 from approx. 80 to 160 pg/ml in healthy volunteers and a significant increase in *FGF21* mRNA expression was observed in skeletal muscle and WAT [53](VII). It was, however, not possible to determine the contribution of FGF21 expressed in skeletal muscle and WAT to plasma FGF21 during the clamp as no liver biopsy was obtained. Insulin has, however, been shown to increase hepatic FGF21 expression *in vitro* by increasing activating transcription factor 4 (ATF4) [427]. The approximately two-fold increases in plasma FGF21 in response to an OGTT or a hyper-insulinemic euglycemic clamp are limited when compared to the ten-fold increase in plasma FGF21 observed in response to PR [60], as discussed below but this does not exclude FGF21 to play an important role in acute glucose handling/insulin sensitivity and macronutrients preference during feeding [114]. The postprandial increase in plasma FGF21 is attenuated in patients with T2D [349] and is potentially also impaired in obese individuals with high plasma FGF21.

Hepatic expression and secretion of FGF21 is increased in response to glucose (ChREBP) and FFA (PPARα), and therefore, it is of specific interest to observe that plasma FGF21 is significantly decreased in insulin deficient STZ treated mice which have high plasma glucose and FFA [17](IX). Also, patients with T1D have decreased plasma FGF21 [428]. This may indicate that insulin is a prerequisite for FGF21 expression [427]. Alternatively, the increase in plasma amino acids in untreated T1D [429] may prevent hepatic FGF21 expression by down regulation of the GCN2 pathway. It is, therefore, of interest to understand the impact of decreased plasma FGF21 in T1D and if people with T1D may benefit from FGF21 treatment [267]. Interestingly, 10 days of PR increases insulin sensitivity (decreases insulin requirement) and lower hepatic glucose production in patients with T1D, potentially mediated by increases in plasma FGF21 in response to protein deficiency [266]. However, recent data do not reveal any association between circulating FGF21 levels and HbA1c in T1D [268].

3.5.2 Regulation of FGF21 in response to amino acid deficiency

The sensing and adaptations to PR and/or deprivation of essential amino acids are vital to counteract lack of proteins, which are essential for survival. In mice, the adaptive response includes hyperphagia, increases in EE as well as upregulation of lipogenic genes and UCP-1 in adipose tissue and down-regulation of lipogenic genes in the liver [430]. Also, an increase in preference of savory-flavored food will ensure enough protein, if available. Furthermore, a reduction in protein oxidation [431] and a decrease in muscle protein degradation [432] serve to prevent nitrogen loss. In contrast to fat and carbohydrate, which can be stored in the body, protein cannot and the compensatory increase in appetite in response to PR will ensure that the “intake target” of proteins is met [433]. Almost 40 years ago, Rothwell and Stock demonstrated that rats fed a low protein diet have increased EE [151]. The increase in EE, in response amino-acid deprivation, has been described to be mediated via induction of CRF in the hypothalamic PVN leading to an activation of the SNS [434]. In response to cold exposure plasma FGF21 is derived from the liver and has been shown to be critical for thermoregulation [148]. FGF21 is also required for upregulation of thermogenesis in the adipose tissue in response to a low protein diet [71]. Thus, FGF21 activates SNS and increases noradrenaline release in BAT [67] both in response to cold exposure and in response to PR. An increase in SNS in the adipose tissue will normally induce lipolysis to fuel UCP-1 [153]. Therefore, it is not obvious why FGF21 treatment acutely decreases plasma FFA [128]. Lipolysis within the brown adipocyte is believed to be required for UCP-1 activity [435], but FFA derived from another cell, for example, WAT to BAT, may also fuel UCP-1 [435]. The decrease in FFA in response to FGF21 treatment may therefore be a consequence of increased FFA uptake into UCP-1-containing adipocytes for utilization.

Plasma FGF21 is significantly increased in response to a KD in mice [61] and FGF21 is a prerequisite for mice to lower BW in response to a KD [436]. However, no increase in plasma FGF21 is observed in humans fed a KD [344, 373, 419, 437]. While the protein content is very low in the KD fed to mice (9.5% of energy from protein versus 23% of energy from protein in chow) [13], the protein content in human KD is balanced or even higher than in a normal diet (up to 35% of energy from protein) [419], indicating that the low protein content in the KD is a major regulator of FGF21 in mice. In agreement with this, plasma FGF21 is increased in response to PR via activation of GCN2 [60]. In mice, plasma FGF21 is increased 10-fold after 15 days of PR (6% energy from protein) [438]. Deprivation of essential amino acids leucine [439] and methionine [440] also increases plasma FGF21 in mice. More data are needed to understand the role of specific amino acids versus general PR in the regulation of FI and EE and if this is mediated by changes in plasma FGF21 [430, 438]. Low protein feeding (10% of energy from protein) has been shown to increase intake of savory-flavored food [441] indicating an increase in protein hunger. The demand for protein is sensed by the hypothalamus via changes in plasma amino acids [442, 443] but other mechanisms potentially involving increases in plasma FGF21 [59, 69, 71, 241] may also be involved. Notably, diets very low in protein (<5%) or diets lacking a single essential amino acid reduce FI. Oppositely, a moderately low protein diet increases FI and protein selection (protein hunger) while a high protein diet suppresses FI [444].

In humans, plasma FGF21 is increased 6-fold after 28 days of low protein intake [60] and, recently, a 6-fold increase in plasma FGF21 was observed in response to 7 days of dietary protein dilution (9% energy from protein) [431]. The pronounced increase in FGF21 in response to PR, may be an integrated signal of high fat, high carbohydrate and low protein intake, which activate three major transcription factors of hepatic FGF21 expression. In 2012, we showed that five days of high-fat feeding increased plasma FGF21 approximately two-fold in healthy volunteers [53](VII) but with the current knowledge, the decrease in protein intake (11% energy from protein) during the five days of high fat feeding may have been the primary driver of the increase in plasma FGF21. The subjects exposed to the high fat diet did not increase BW and a slight increase in EE was observed; however, plasma FGF21 did not correlate significantly with increases in EE [53](VII). Overeating of low protein diets for 28 days caused a 6-fold increase in plasma FGF21 but did not increased BW despite no increase in EE [445]. Plasma FGF21 has, however, been shown to correlate with 24 hours EE in subjects fed a low protein diet for 24 hours [272] and subjects who failed to increased plasma FGF21 were more prone to BW gain [272]. Interestingly, an eight-fold increase in plasma FGF21 has been observed in humans in response to three days of carbohydrate rich diet feeding, despite no change in protein content [135] demonstrating that FGF21 also is involved in the metabolic adaptation in response to a high carbohydrate diet. In summary, plasma FGF21 is robustly increased by PR in both mice and humans [305, 431]. FGF21 is required to increase hyperphagia and EE in response to PR in mice [60]. Notably, while rodents metabolize and dissipate a substantial portion of over-ingested energy, humans mainly store energy [446]. The ability to store fat has been a great advantage for humans preparing for time of sparse food supply and evolutionally this phenotype has been favored [447]. It is therefore, interesting to note that individuals potentially lacking FGF21 have lower fat mass, visceral obesity and insulin resistance, indicating a defect in the ability to store subcutaneous fat [214]. As protein is a minor component (15% of energy from protein) of the total diet and cannot be stored like fat and glucose, a small decrease in protein content results in overeating and has been hypothesized to cause obesity (the protein leverage hypothesis) [433, 448]. However, more data are required to understand the role of FGF21 in hyperphagia, food preferences and EE in humans.

3.6 The role of FGF21 in fasting versus protein restriction

Long term fasting/starvation and PR decrease BG, plasma insulin and IGF-1 and increase appetite and food-seeking behavior. However, while fasting/starvation decreases EE, insulin sensitivity and induces lipolysis, PR increases EE, insulin sensitivity [305] and promotes adipogenesis (decreases lipolysis). Furthermore, the excess energy intake (carbohydrate and fat) in response to PR (high energy intake) is either utilized (increase in EE) or stored as TG in adipose tissue. The increase in appetite during starvation is driven by a decrease in plasma leptin, which does not occur as a response to PR in mice [449]. However, the soluble leptin receptor is increased [449] and similar observations are observed in FGF21-treated mice [170], thus the lower free fraction of leptin may increase appetite. However, other mechanisms including decreases in plasma amino acids [442] and direct effect CNS effects of FGF21 [70] may also be involved in PR-induced hyperphagia and macronutrient preferences. Thus, while long-term fasting/starvation and protein restriction have overlapping metabolic phenotypes, such as reduction in plasma insulin and IGF-1, they are distinct metabolic states as energy intake is not limited during PR.

Fasting, PR and FGF21 treatment decrease plasma insulin, but while an increase in lipolysis is observed in response to fasting, a decrease is observed in response to FGF21 treatment [128]. A decrease in lipolysis will decrease hepatic acetyl-CoA and hepatic gluconeogenesis [129]. This may be an important adaptation [151, 152] to spare amino acids used for gluconeogenesis during PR, while an increase in gluconeogenesis is required in times of starvation. Glucagon, which is increased in response to fasting and high plasma amino acids [450] to promote gluconeogenesis and ureagenesis [451, 452], should therefore decrease in response to PR. Plasma glucagon is decreased in response to FGF21 treatment [93] and as all plasma amino acids are decreased in rats fed a low protein diet (8,7% versus 17% energy from protein) [453] glucagon is also expected to decrease in response to PR. Finally, both fasting and PR decrease plasma IGF-1, but in contrast to fasting, which increases plasma GH, PR decreases GH release in rats [454, 455]. In humans, the dietary intake of protein is also a major determinant of plasma IGF-1 compared to calorie restriction [456] and notably plasma FGF21 was negatively associated with plasma IGF-1 in non-diabetic subjects in a multiple regression analysis [343]. Fasting induces hepatic GH resistance by down regulation of the hepatic GH receptor, in contrast to PR, which does not alter hepatic GH binding [457]. It is not clear why the low plasma IGF-1 during PR does not induce GH release but changes in hypothalamic growth hormone releasing protein and somatostatin release as well regulation of IGF binding proteins [458] may be involved. Ghrelin, which stimulates GH, is released in response to fasting [459], but potentially not in response to PR and FGF21 treatment does not change plasma ghrelin in NHP [9]. The increase in GH in response to fasting is important to induce lipolysis, fat oxidation and insulin resistance [368] and blockage of GH during fasting reverses insulin resistance and decreases fat oxidation [14]. Interestingly, FGF21 treatment decreases GH release in obese pigs [92](II) and rats (B. Andersen, unpublished observations), while Inagaki, et al. report an increase in GH (one time point) in response to FGF21 treatment of mice [224]. The potential decrease in GH release in response to PR [454, 455] and in response to an OGTT [460] may facilitate glucose uptake and increase insulin sensitivity. Thus, blockage of the PR-induced FGF21 release and the postprandial increase in plasma FGF21 response may increase GH and decrease insulin sensitivity. More data are required to substantiate this hypothesis and the hypothesis can furthermore be challenged by human genetic data. The allele rs838133 which is described to be a loss of FGF21 function variant is associated with visceral obesity and thereby it is unlikely that these individuals have high plasma GH.

3.7 Summary physiology

Plasma FGF21 is increased under several pathological conditions such as T2D and obesity. Plasma FGF21 is also increased in response to an HFD in mice, monkeys and humans, which may be secondary to low protein intake. Plasma FGF21 is increased in mice in response to fasting and this may also involve decreases in plasma amino acids. In humans, FGF21 activity may decrease with fasting due to down regulation of the FGF21 receptor complex in WAT [15, 57](V), supporting the hypothesis that FGF21 is not required or involved in the adaptive response to fasting in humans. The decrease in FGF21 activity is associated with decreases in EE [15], increases lipolysis and decreases in glucose and TG uptake in the adipose tissue [370]. The late increase in plasma FGF21 observed in humans in response to fasting may be associated with decreased in plasma amino acids and an eventual demand for proteins. The decrease in plasma FGF21 after three days of fasting in obese subjects, may on the other hand, be due to metabolic improvement (insulin resistance and decreases in hepatic fat). Regulation of FGF21 and the FGF21 receptor complex in WAT in response to fasting in humans is shown in Figure 8.

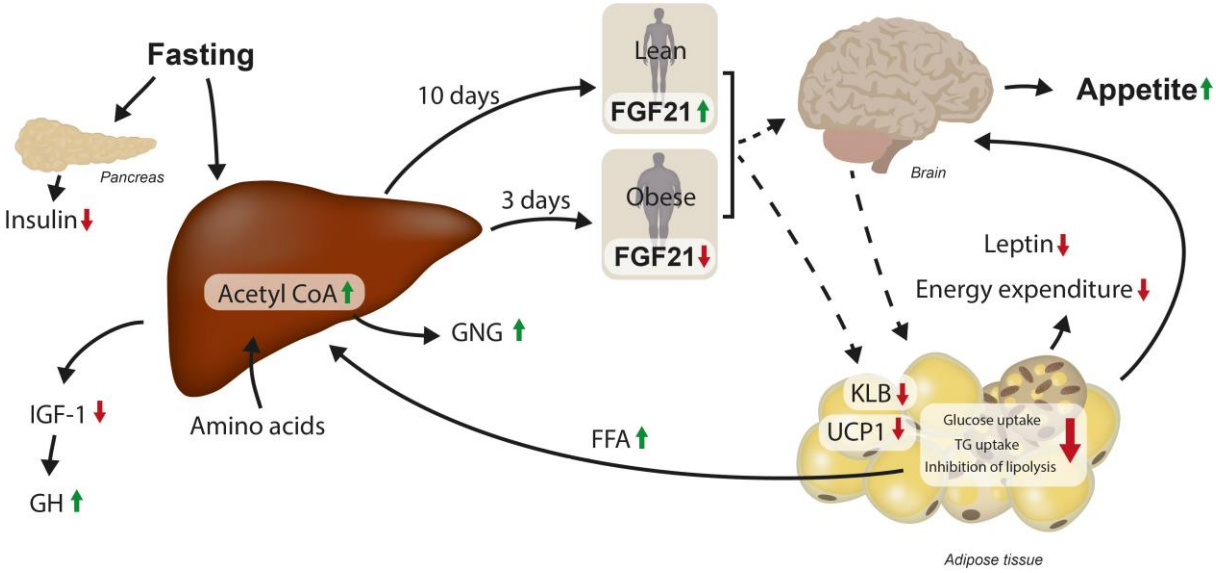

FIGURE 8 REGULATION OF FGF21 ACTIVITY IN RESPONSE TO FASTING IN HUMANS

In lean subjects, FGF21 is increased in response to 10 days of fasting, while 3 days of fasting decreased plasma FGF21 in obese subjects. KLB and FGFR1c and thereby potentially also FGF21 activity is decreased in WAT in response to fasting. A decrease in FGF21 activity will allow flow of FFA from the adipose tissue, decrease glucose uptake into the adipocytes and decrease EE. In response to fasting plasma insulin and IGF-1 decrease, and a decrease in plasma IGF-1 induces release of GH. In response to fasting plasma leptin is decreased driving an increase in appetite.

The increase in plasma FGF21 in humans in response to PR [53, 60](VII) and in response to an OGTT [247, 425](VIII) may acutely lower GH to induce insulin sensitivity [114, 349], promoting glucose oxidation and energy storage in the adipose tissue [4, 166]. This hypothesis does, however, require more data to be substantiated and the effect on FGF21 on GH release needs to be confirmed. Furthermore, as FGF21 is tightly regulated by FFA, glucose and amino-acids, full FGF21 activity (EE and glucose and lipid uptake into adipose tissue) may only occur if protein intake is restricted along with a high intake of carbohydrate and fat [305]. The proposed FGF21 response to PR and high glucose is shown in Figure 9.

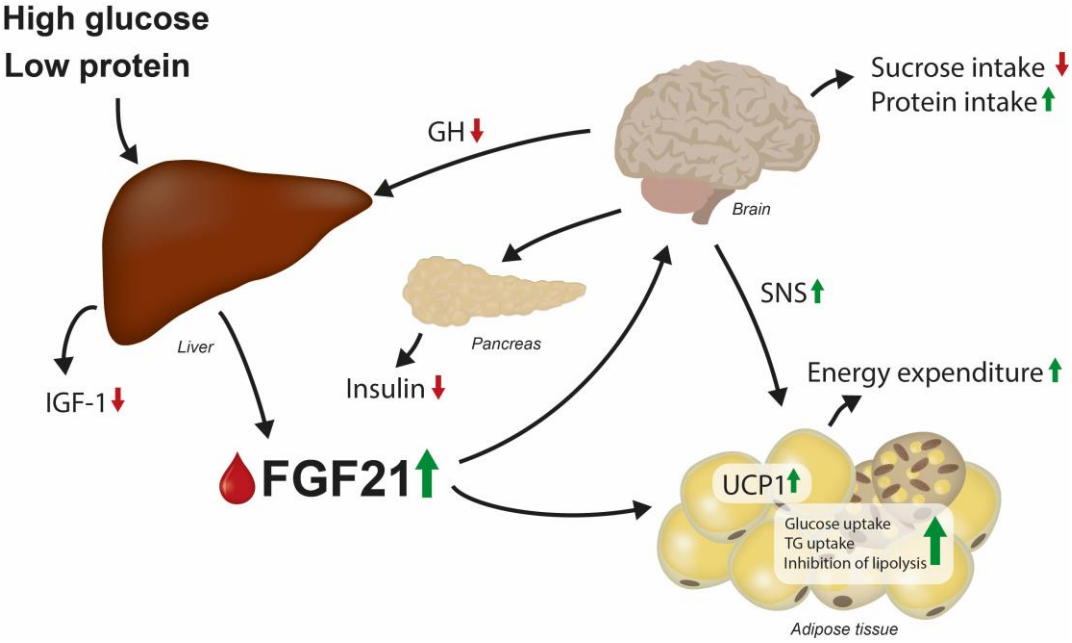

FIGURE 9 REGULATION OF FGF21 ACTIVITY IN RESPONSE TO PR AND OGTT

In response to high glucose and low amino acid levels in plasma, FGF21 is released from the liver. FGF21 increases glucose and lipid uptake in the adipose tissue for storage and utilization. FGF21 increases expression of UCP-1 in the adipose tissue and EE is induced by the SNS at least in rodents. FGF21 decreases plasma FGF21 which decreases hepatic mTOR activity. FGF21 may also potentially decrease GH release from the pituitary. A decrease in plasma GH will increase glucose oxidation and increase insulin sensitivity. FGF21 stimulates protein intake and decreases sucrose intake to meet the demand for proteins.

3.7.1 From physiology to pharmacology

In response to a low protein diet, FGF21 is released from the liver and food preferences are altered. An increase in protein intake [69] and a decreased preference for sucrose [59] will ensure the body's protein demand. If food is available but low in protein content, overeating will increase the intake of carbohydrate and fat and the increase in plasma FGF21 will facilitate uptake of glucose and lipids into the adipose tissue, where it utilized or stored as subcutaneous fat. Simultaneously, plasma insulin is decreased preventing hepatic lipid accumulation. The phenotypic response to amino-acid deprivation is thereby mimicked by FGF21 treatment and patients with obesity, T2D and NASH may potentially benefit from FGF21 treatment, which will increase insulin sensitivity, lower plasma lipids and decrease ectopic fat accumulation in liver and skeletal muscle. However, while PR lowers BW in mice [60, 444] a low protein diet may drive obesity in humans [433, 448].

The proposed hypothesis linking low GH to the insulin sensitizing effect of FGF21 needs to be established. Moreover, IGF-1 is lowered in mice, rats, pigs and monkeys treated with FGF21, while plasma IGF-1 was not decreased in humans treated with an FGF21 analogue [9]. As FGF21 treatment may increase protein intake in humans as observed in mice [69], this may counteract the potential inhibitory effect of FGF21 on GH release. This may prevent loss of lean mass [461] but may impair the BG lowering and insulin sensitizing effect of FGF21. Several longitudinal studies have associated high protein intake with increased mortality, as reviewed by Simpson et al. [462] and low protein intake and high carbohydrate intake, presumably causing an increase in plasma FGF21, is beneficial for longevity and late life health [317]. Conversely, a diet low in protein trends to drive overconsumption of total energy leading to obesity and the two scenarios need to be balanced. Interestingly, GH release is decreased in NASH [463] and critically ill patients [464], potentially due to increases in

FGF21. However, GH replacement therapy increases morbidity and mortality in critically ill patients [465] and, therefore, the FGF21-mediated decrease in GH release may be important to prevent oxidative stress [466]. In mice FGF21 protects toward sepsis-induces toxicity [467] and FGF21 may potentially also improve the outcome of other chronic diseases [468-470].

In conclusion, FGF21 does not play a significant role in response to fasting in humans in contrast to previous suggestions, but it clearly plays an important role in the adaptations to PR. Thus, insight into the physiological role has facilitated the understanding of the pharmacological effect of FGF21 observed across species. The strong effect of FGF21 on food preferences and the ability of high fat feeding to increase EE in mice and monkeys explain some of the translational challenges that have been encountered during FGF21 drug development. No single animal model may have predicted human efficacy better than any other but based on the novel insight into the physiological role of FGF21, the pre-clinical studies should have addressed the effect of food preferences earlier.

4 PART III-a: Overlapping pharmaco-dynamics effects of molecules with distinct physiological effect

4.1 Introduction

An increasing number of publications show increases in plasma FGF21 in response to various stimuli and the metabolic efficacy of these stimuli is often discussed to be FGF21-mediated. One such stimulus is provided by the hormone glucagon. Glucagon in released from the pancreatic α-cells in response to low glucose and high amino-acids in contrast to FGF21, which is released from the liver in response to high glucose and low amino-acids levels. The connection between these two hormones is therefore not clear.

FGF19 and FGF21 have been shown to have overlapping activities in mice despite distinct physiological functions of the two molecules. FGF19 regulates bile acid homeostasis while FGF21 coordinates EE, lipid and glucose metabolism. It is unclear why overlapping effects are observed in mice treated with rhFGF19 and rhFGF21.

4.2 Association between Glucagon and FGF21

Glucagon is expressed in the α-cells of the pancreas and increases BG by increasing hepatic glucose production [357]. Plasma glucagon is increased in subjects with T2D and neutralization of glucagon action was proposed as an anti-diabetic therapy [471]. Glucagon antagonism was actively pursued as drug targets for the treatment of T2D with limited success [472]. Glucagon increases EE in mice and within the last decade glucagon receptor and GLP-1 receptor co-agonists have been investigated for their potential to treat obesity and T2D [473]. FGF21 has also, as discussed previously, been tested for its ability to lower BG and BW [5]. It was therefore highly interesting to observe that glucagon is unable to increase EE in the FGF21 KO mice [382, 474]. Furthermore, glucagon increases hepatic expression of FGF21 and plasma FGF21 is increased 2-3-fold in mice treated with glucagon [382, 474, 475]. In contrast, plasma FGF21 is increased 25-fold in the glucagon receptor (Gcgr) KO mice compared to WT littermates [17](IX). Glucagon and FGF21 are oppositely regulated, as glucagon is released from α-cells during hypoglycemia [476] and in response high plasma amino acids [477], while hepatic FGF21 is increased in response to high glucose [58, 247, 478](VIII) and during PR [53, 60](VII). It is therefore of high interest to understand why both glucagon agonism and antagonism

(Gcgr KO) increase plasma FGF21, and if glucagon and FGF21 have overlapping pharmacological effects in mice and man.

4.3 Glucagon

Glucagon is a 29 amino acid peptide hormone secreted by the α-cells in the pancreas [479]. Glucagon acts as a counter-regulatory hormone to insulin, such that glucagon is secreted during hypoglycemia while insulin is secreted in response to hyperglycemia. Recombinant human glucagon (e.g. GlucaGen) is an approved therapy for severe diabetic hypoglycemia/ketoacidosis and sc administration of glucagon increases plasma glucose immediately [480]. The enteroendocrine L-cells in the intestine may also express glucagon [481] and it is released both from both the intestine and the pancreatic α-cells [477, 482] in response to high protein feeding. A protein-rich meal also stimulates the release of insulin and hypoglycemia occurs if glucagon release is blocked [483, 484]. All amino acids can stimulate glucagon secretion to various degrees [483].

Glucagon acts through the glucagon receptor, a G protein-coupled receptor [485, 486]. Glucagon receptors are highly expressed in hepatocytes and their activation stimulates hepatic glucose production [487]. Initially, glycogen will be converted to glucose by activation of glycogen phosphorylase. During prolonged fasting gluconeogenic amino acids become important substrates for gluconeogenesis [476, 488]. In hepatocytes, glucagon also stimulates β-oxidation and ketogenesis [489] while it decreases DNL [490, 491]. The decrease in DNL and increase in β-oxidation significantly decreases hepatic lipid accumulation [492]. In addition, glucagon stimulates degradation of LDLc in hepatocytes [493]. Glucagon is also an important regulator of amino acid metabolism [211] and it stimulates ureagenesis [494], which removes ammonia from the liver [495]. Notably, patients with glucagonomas are not clinically presented with hyperglycemia, but suffer from necrolytic migratory erythema caused by hypoaminoacidemia [496] and BW loss [497]. Conversely, individuals with a loss of function mutation in the glucagon receptor are not hypoglycemic but display α-cell hyperplasia and pancreatic swelling (because of α-cell hyperplasia) [211] due to hyperaminoacidemia [498].

Glucagon is additionally described to have activities in the CNS [499] and a direct effect of the appetite controlling neurons has been proposed [499]. Interestingly, of all macronutrients, protein, which increases glucagon release, is short-term the most satiating macronutrient per calorie and high protein intake is central to many weight-loss strategies [500]. Furthermore, glucagon receptors are expressed in the adipose tissue [501, 502] where glucagon increases lipolysis [503] but species-specific differences have been observed [502]. The increased flow of FFA from the adipose tissue can be utilized as energy in skeletal muscle and liver and hepatic FFA can via increases in acetyl-CoA and glycerol stimulate gluconeogenesis [129]. Glucagon also mimics the action of CRF and administration of glucagon increases ATCH and glucocorticoids in humans [504]. This further stimulates proteolysis of skeletal muscle and hepatic gluconeogenesis [505]. Moreover, CRF has also been observed to be involved in the induction of EE [506]. In mice the glucagon-induced EE is mediated by an increase in non-shivering thermogenesis and upregulation of UCP-1 in the adipose tissue [475]. Like FGF21 [147] glucagon is released in response to cold exposure in rats and in humans [507] and glucagon has been shown to play an important role in the adaptive thermogenesis in BAT in mice [475]. Recent studies have, however, shown that induction of EE by glucagon does not require expression of glucagon receptors in the BAT [474] and glucagon also stimulates EE in UCP-1 KO mice but the effect was found to be partly FGF21 dependent [382, 474, 508]. Glucagon also stimulates EE in pigs with no UCP-1 [509].

The liver is pivotal for the glucagon thermogenic effect and glucagon does not increase EE in liver-specific Gcgr KO mice [508]. The effect on glucagon of EE in mice has also be shown to be dependent

on an increase in bile acids [474, 508]. In healthy volunteers, a glucagon infusion has been reported to increase EE by 15% without activation of BAT [510], while other studies fail to observe any effect on resting EE after a glucagon infusion [511]. The effect of glucagon on EE in humans has recently been revived by Kleinert, et al. [512] and the ability of glucagon to increase EE depends on the metabolic state (pre-prandial versus post-prandial) and glucagon does not increase EE when co-infused with insulin [513]. In untreated T1D patients [429] and in patients with glucagonoma [514] with a high glucagon/insulin ratio, an increase in protein turnover is observed and whole-body protein breakdown is increased. Protein turnover (degradation into urea or conversion into glucose) are energy-demanding processes and these been speculated to contribute to the increase in EE [429, 497]. Finally, loss of glucose in the urine also contributes to a negative energy balance in insulin deficient T1D with high plasma glucagon and high BG.

4.4 Overlapping and distinct effects of glucagon and FGF21

Glucagon and FGF21 have been shown to have overlapping actions on EE in mice [6, 7, 382, 474] and both glucagon [515] and FGF21 [115] have been described to increase oxygen consumption and glucose uptake in adipocytes *in vitro*. However, glucagon and FGF21 have opposite actions on BG. Glucagon acutely increases BG [476, 481] while FGF21 decreases BG in diabetic mice [4, 18](X). Moreover, while FGF21 inhibits lipolysis [56] glucagon increases lipolysis at least in rats [503] Glucagon stimulates ureagenesis [494, 516] and therefore the Gcgr KO mice have increased plasma amino acids [517]. The effect of FGF21 on amino-acid turnover is unknown but FGF21 may lower amino acid oxidation and decrease ureagenesis as observed during PR [431]. Interestingly, both glucagon [518] and FGF21 [132] decrease hepatic mTOR activity, potentially through a reduction in hepatic amino acids. A decrease in hepatic mTOR lowers hepatic lipid accumulation and decreases plasma TG and cholesterol [519, 520].

Fasting regulates the GH/IGF-1 axis [362] and due to hepatic GH resistance, hepatic IGF-1 and IGFBP3 are not induced [521]. The decrease in IGF-1 further increases GH release from the pituitary, which stimulates lipolysis [377] and hepatic glucose production [522]. Both glucagon [523] and FGF21 [224] have been shown decrease plasma IGF-1 bioavailability. However, while glucagon enhances GH release [524] FGF21 may decrease GH release [92](II). The potential differential regulation of GH release by glucagon and FGF21 will result in opposite effect on lipid oxidation, insulin sensitivity and BG. Glucagon is furthermore increased in response to various stresses including trauma, burns, surgery and sepsis as reviewed by Jones et al. [525]. Similar observations have been made with FGF21 [290, 526-528]. In addition to glucagon's effect on EE, the peptide has been shown to decrease FI in mice [529] in contrast to FGF21, which increases FI [6]. Both glucagon [504] and FGF21 [130] have been described to activate the HPA axis in mice and interestingly, glucagon does not increase EE in adrenalectomized and thyroidectomized rats [530].

4.5 Regulation of FGF21 by Glucagon

Glucagon has been shown in mice to increase hepatic FGF21 expression by activation of AMPK, cyclic adenosine monophosphate (cAMP) and PPARα [531, 532]. Glucagon stimulates plasma FGF21 three-fold and the glucagon/GLP-1 analogue IUB288 increases FGF21 two-fold in mice [382]. However, the IUB288 glucagon analogue has a weak and acute hyperglycemic action in vivo despite its extended t½, while at the same time showing potent and long lasting effects on BW and cholesterol compared to glucagon [533]. Therefore, the GLP-1 part of IUB288 (despite being a weak GLP-1 receptor agonist) may also contribute to the BW lowering effect of IUB288 and, interestingly, FGF21 also contributes to GLP-1-mediated weight loss in mice [534]. Furthermore, liraglutide a long-acting GLP-1 receptor

agonist, increases plasma FGF21 in mice [535, 536] potentially by activation of invariant natural killer T (iNKT) cells [534]. Therefore, in mice, both glucagon and GLP-1 receptor agonism increase plasma FGF21. In humans, glucagon increases FGF21 two to three-fold 60-180 min after injection depending on the study [382, 474, 532]. Additionally, exercise-induced FGF21 release has been shown to be associated with and potentially secondary to increases in splanchnic glucagon [537, 538].

Through activation of hepatic ChREBP, glucose is a strong inducer of FGF21 expression [58] and secretion [247](VIII). Therefore, the glucagon-induced increase in hepatic glucose production may also increase hepatic FGF21. The contribution of hepatic glucose production to FGF21 secretion can be studied using a glycogen phosphorylase [539] or glucose-6-phosphatase inhibitor [540] or by use of the ChREBP ko mice [541] treated with glucagon. Another indirect mechanism by which glucagon-stimulated hepatic FGF21 occurs may be via activation of GH release [523]. GH has been shown to stimulate hepatic FGF21 production secondary to induction of lipolysis [56]. Therefore, as depicted in Figure 10 several mechanisms secondary to activation of the glucagon receptor may be involved in the observed increase in plasma FGF21 in response to glucagon [382, 474, 532]. In contrast to the stimulatory effect of glucagon on FGF21 secretion, other studies have shown that glucagon decreases plasma FGF21 in diabetic rats [542]. This may be correlated to a decrease in glucose, as the glucagon analogue used in the study was given together with insulin. Notably, the high glucagon/insulin ratio observed in T1D [267] should furthermore increase plasma FGF21, but the opposite is observed [17](IX). It is also noteworthy that FGF21 is not increased in response to glucagon during fasting [342](VI) but only increased in response to pharmacological doses of glucagon.

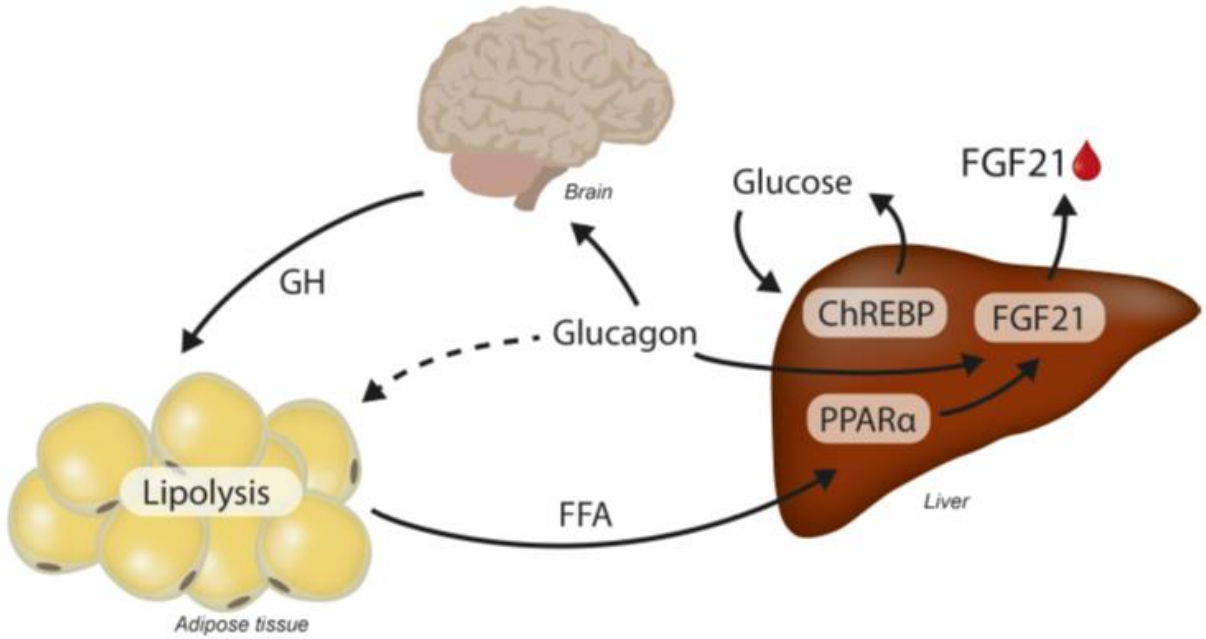

FIGURE 10 DIRECT AND INDIRECT REGULATION OF FGF21 BY GLUCAGON

Glucagon treatment increases plasma FGF21 two to three-fold. Glucagon stimulates FGF21 expression in hepatocytes but may also induce hepatic FGF21 expression by stimulation of hepatic glucose production. GH which stimulates lipolysis and thereby FGF21 are also increased in response to glucagon treatment. The direct effect of glucagon on adipose tissue may depend on the species (observed in rat adipocytes) and therefore illustrated by a dotted line.

4.6 Regulation of FGF21 by lack of glucagon

In contrast to the modest regulation of FGF21 by glucagon and IUB288, lack of glucagon action in the Gcgr KO mice significantly increased plasma FGF21 25-fold [17](IX). Remarkably, despite use of the Gcgr KO mice in previous publications, no report of increased plasma FGF21 has been mentioned but interestingly, FGF21 levels have been normalized to 100% in the glucagon-treated Gcgr KO mice [382]. Mice lacking Gcgr display α-cell hyperplasia [543] and as *Fgf21* gene expression is observed in the pancreas of mice [21] we hypothesized that FGF21 was increased in the pancreas of Gcgr KO mice. As seen in Figure 11(E-H)) α-cells (glucagon positive cells) in the Grgr KO mice stained positive for FGF21 which may explain the large increase in plasma FGF21 in these mice. In the wt mice FGF21 was co-localized with both insulin- and glucagon-expressing cells, Figure 11(A-D). In the liver, mTOR has been shown to increase plasma FGF21 [544] and activation of mTOR secondary to increases in plasma amino-acids [545] in α-cells may also increase FGF21 expression in these cells. Plasma FGF21 is suggested to be mainly liver-derived [114] and the pancreatic FGF21 it not believed to contribute to plasma FGF21 [328]. However, with the relatively modest expression of FGF21 in liver and adipose tissue in the Gcgr KO mice [17](IX), the endocrine pancreas likely contributed to plasma FGF21 in these mice.

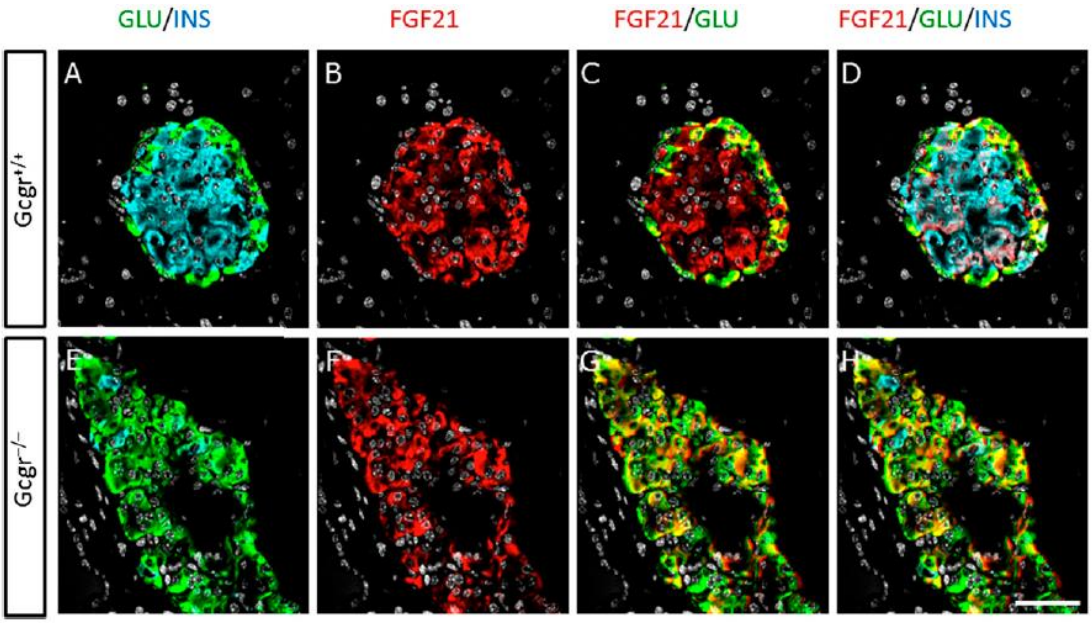

FIGURE 11 MOUSE ISLETS FROM WT AND GCGR KO MOUSE STAINED FOR GLUCAGON, INSULIN AND FGF21 (FAT FROM PUBLICATION IX)

Localization of FGF21 in wild-type and Gcgr KO mice (Grcg -/-). Immunohistochemical stains of wt mice (A-D) and Gcgr KO mice (E–H) pancreatic sections using antiserum against FGF21 (red) and insulin (blue) and Ab against glucagon (green). Nuclear staining was done using DAPI (gray). Scale bar, 50 µm. INS: insulin and GLU: Glucagon.

A two-fold increase in *Fgf21* mRNA expression was observed in the livers of the Gcgr KO mice which may be associated with changes in amino-acids metabolism. Moreover, an increase in plasma corticosterone is observed in the Gcgr KO mice [543] and, as discussed for glucagon agonism, glucocorticoids may increase hepatic FGF21 expression [319]. Conversely, the high plasma FGF21 may also be responsible for the increase in plasma corticosterone observed in the Gcgr KO mice [32]. An increase in *Fgf21* mRNA was also observed in WAT in the Gcgr KO mice and this increase might be

secondary to FGF21-induced “beiging” of adipocytes [147]. It is, however, unlikely that FGF21 released from adipose tissue will enter circulation [114]. The increases in pancreatic proglucagon and subsequent GLP-1 release from α-cells in the Gcgr KO mice [543] may also, as mentioned previously, increase plasma FGF21 [534, 535]. Notably, no blood glucose-lowering effect was observed in STZ-treated mice lacking both the glucagon and GLP-1 receptors (Gcgr and Glp1r double KO mice) while hyperplasia of a-cells and increases in plasma amino-acids were maintained [546]. Unfortunately, plasma FGF21 was not determined in these double KO mice. As shown in Figure 12 plasma FGF21 in the Gcgr KO may arise from pancreas, liver and adipose tissue

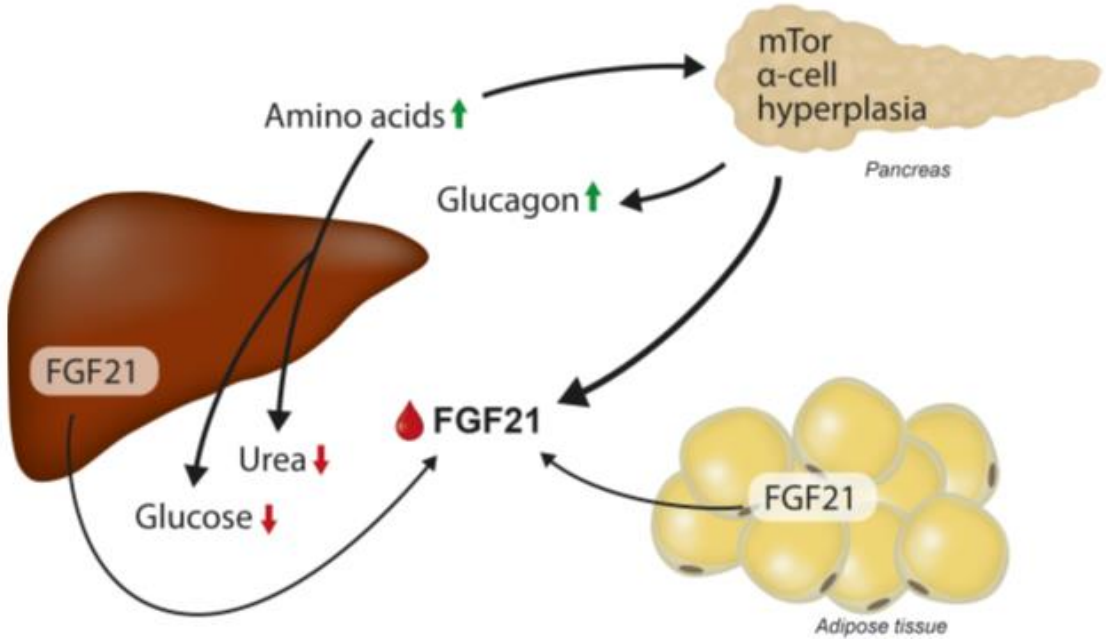

FIGURE 12 REGULATION OF FGF21 IN THE GCGR KO MICE

Plasma FGF21 is highly increased in the Gcgr KO mice. Based on immunobiological staining FGF21 is highly expressed in the α-cells of the pancreas. FGF21 may however still be released from the liver.

4.7 Glucagon receptor deficiency and the anti-diabetic effect

The global Gcgr KO mice were created using a gene targeting strategy deleting exon 3-6 of the murine *Gcgr* gene by homologous recombination [543]. The KO mice are born at normal term and display normal growth rates [543]. Postnatally an enlargement of the pancreas with hyperplasia of the α-cells is observed and supra-physiological glucagon levels are present [543, 545, 547, 548]. The Gcgr KO mice display slightly lower BG throughout the day, despite similar insulin levels [543], and in response to a hyperinsulinemic-euglycemic clamp glucose infusion rate is significantly increased in the Gcgr KO mice compared to wt mice [549]. The Gcgr KO mice have normal BW but reduced adiposity and the KO mice are resistant to diet-induced obesity, however, plasma LDLc and TG levels are increased [543].

In 2011 Lee, et al. showed that multiple high doses of the β-cell toxin STZ does not cause diabetes in the Gcgr KO mice [550]. The suggested mechanism behind the anti-diabetic effect was lack of glucagon action, which would prevent hepatic glucose production [550]. However, as described above, the Gcgr KO mice have elevated plasma FGF21 [17](IX) and GLP-1 [549, 551]. Therefore, we set out to antagonize FGF21 and GLP-1 to understand the contribution of these two hormones to the anti-diabetic effect observed in the STZ-treated Gcgr KO mice. As seen in Figure 13, the improvement

in glucose tolerance, observed in the Gcgr KO mice, was abolished by antagonizing both FGF21 and GLP-1 without changing plasma insulin [17](IX).

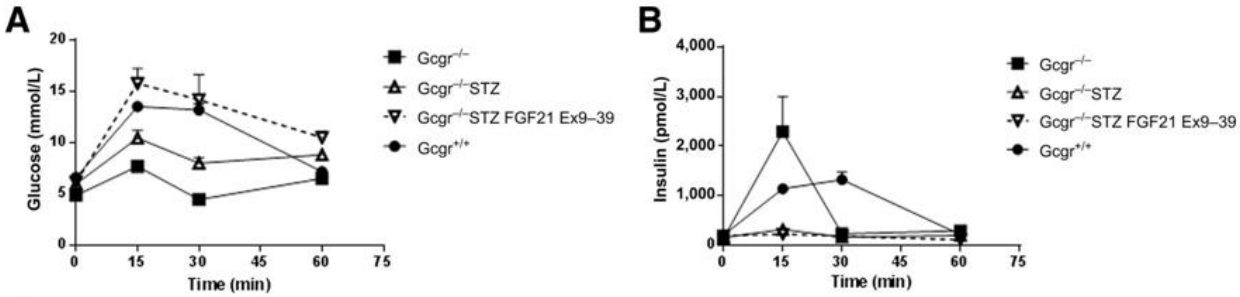

FIGURE 13 GLUCOSE TOLERANCE TEST IN WT AND GCGR KO MICE TREATED WITH FGF21 AB AND EX9-39 (DATA FROM PUBLICATION IX)

Effects of combined FGF21 neutralization and GLP-1 receptor antagonism on oral glucose tolerance in insulin-deficient Gcgr KO mice. Injections of FGF21 neutralizing Abs and Ex9-39 were given 5 hous and 10 minutes prior to the OGTT, respectively. Plasma glucose (A) and insulin (B) excursions during the OGTT (75 mg/mouse) in wt **(***Grcg+-/+), Gcgr KO* (*Grcg -/-), Gcgr KO STZ, and Gcgr KO STZ FGF21 Ab/Ex9-39–treated mice.*

Therefore, the improved glucose tolerance/anti-diabetic of the *Grcg* KO is not driven by lack of glucagon but rather by secondary increases in plasma GLP-1 and FGF21 [17](IX) as schematically shown in Figure 14, supporting the hypothesis that the high plasma FGF21 contributes to the anti-diabetic effect in these mice. Intriguingly, GLP-1 receptor agonism has been shown to increase the expression of the FGF21 co-receptor KLB in the adipose tissue of mice [338] and as neither GLP-1 nor FGF21 antagonism alone were able to abolish the positive effect on BG in the STZ-treated Gcgr KO mice, a potentiating interaction between FGF21 and GLP-1 may occur.

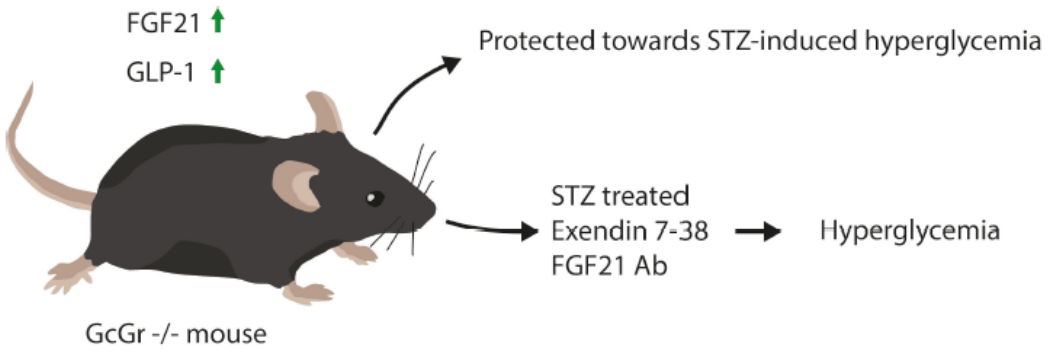

FIGURE 14 SCHEMATIC OVERVIEW OF THE ANTI-DIABETIC EFFECT OF GLP-1 AND FGF21 IN STZ-TREATED GCGR KO MICE

The increase in insulin sensitivity observed in the Gcgr KO mice as well as the protective effect towards diet-induced obesity may also both be caused by the high plasma FGF21 [6, 7]. It would be interesting to observe if these effects persist if the Gcgr KO mice were crossed with the FGF21 KO

mice. The increase in plasma cholesterol and TG observed in the Gcgr KO mice [543] is, however, not mitigated by a 25-fold increase in plasma FGF21, despite the strong plasma lipid lowering effect of FGF21 [4, 6, 7] and more studies are required to understand why FGF21 does not decrease cholesterol and TG in the Gcgr KO mice. However, increases in plasma amino acids [211] may overrule the inhibitory effect of FGF21 on hepatic mTOR activity [171] and increases in hepatic mTor activity will increase plasma TG and cholesterol [172, 520]. Thus, future studies addressing the role of hepatic mTOR activity (e.g., rapamycin treatment of the Gcgr KO mice) to plasma lipids in the Gcgr KO mice are needed.

4.8 Therapeutic potentials of Glucagon antagonism and Glucagon/GLP-1 receptor agonism

Neutralization of glucagon's hyperglycemic effect has been investigated as a therapeutic approach to lower blood glucose for decades [472] and various approaches to inhibit glucagon action like small glucagon receptor antagonist [552, 553], glucagon antibodies [554] and small interfering (si)RNA of the glucagon receptor [555] have been investigated. Several approaches were tested in humans and in one clinical study a small molecule glucagon receptor antagonist decreased glycosylated Hb1Ac 1.5% after 24 weeks of treatment [556]. The development of glucagon receptor antagonists was, however, stopped due to increases in plasma liver enzymes and plasma LDLc [472]. The increase in liver enzymes may have been secondary to increases in hepatic ammonia due to decreases in ureagenesis [557] but may also be secondary to increases in hepatic steatosis [558]. Additionally, α-cell hyperplasia which is observed in the Gcgr KO [559] will potentially also occur in humans [545]. Increases in BP have also been observed in humans treated with glucagon receptor antagonist [560]. Thus, the therapeutic benefit of lowering BG was outweighed by several side effects. It is currently not known if glucagon antagonism increases plasma FGF21 in mice or humans.

Glucagon and GLP-1 receptor co-agonists are currently in clinical trials for the treatment of T2D and obesity [561-563]. The purpose of a dual GLP-1/glucagon receptor agonist is to target both the glucagon and the glucagon-like peptide-1 (GLP-1) receptors to obtain a greater weight loss than obtained by GLP-1 alone. Too much glucagon action will, however, induce hyperglycaemia and the "amount" of glucagon will have to be counter balanced by GLP-1, which increases insulin release and thereby lowers BG. Conversely, the beneficial effect of glucagon on hepatic lipid oxidation, ureagenesis, plasma lipids, EE and FI is expected to be preserved [564]. Thus, the ratio between glucagon/GLP-1 is of crucial importance. In monkeys, a GLP-1/Glucagon receptor co-agonist (MEDI0382) decreases BW, but unfortunately no data on FI, EE or plasma FGF21 were published [565]. Initial clinical trials show that a balanced glucagon/GLP-1 co-agonist (MEDI0382) decreased BG, appetite and BW in humans with T2D [562] compared to placebo, but further clinical trials are required to establish the beneficial effect in a larger population. It is not known if EE or plasma FGF21 was increased in humans treated with MEDI0382. The pharmacological effect of FGF21 in pre-clinical and humans have been described previously and future clinical studies are also required to establish the metabolic benefits of FGF21 treatment in humans.

4.9 Conclusion and future aspects

In conclusion, glucagon and FGF21 have overlapping effects on EE and plasma lipids in mice but also distinct effects on glucose and amino acid metabolism. The stimulatory effect of glucagon on plasma FGF21 is minor, but FGF21 is required for glucagon's ability to induce EE in mice, while it is unknown if FGF21 is also involved in the lipid lowering effect of glucagon. The anti-diabetic effects observed in STZ-treated Gcgr KO mice were shown to be mediated by GLP-1 and FGF21 [17](IX) and the effects were not observed in mice lacking both the GLP-1 and glucagon receptor [546]. The large increase in

plasma FGF21 in the Gcgr KO mice [17](IX) may be involved in the increased insulin sensitivity and the resistance towards diet-induced obesity observed in the Gcgr KO mice, but more data are required to determine the contribution of FGF21 to these effects. Despite the large increases in plasma FGF21 in the Gcgr KO mice, plasma cholesterol and TG were increased, indicating that the high plasma amino-acids [520] via hepatic mTOR may override the lipid lowering effect of FGF21. It is still not clear why FGF21 is required for glucagon to induce EE in mice and additional studies using, for example, β-blockers or adrenalectomized mice, will increase our understanding of the pathway connecting glucagon to FGF21.

It will also be of high importance to understand if glucagon/GLP-1 receptor co-agonists stimulate EE in NHPs or in humans. An initial clinical study showed a decrease in appetite in humans treated with MEDI0382 [562]. As discussed previously, FGF21 affects dietary preference, increasing protein intake [69] and decreasing sucrose intake in mice [59], and therefore it will be of great interest to observe if the opposite effect (decrease in protein and increase in sucrose intake) is observed in response to glucagon treatment. Dysregulation of amino-acids metabolism affects glucose, lipid and energy metabolism by a complex regulation of mTOR, glucagon and FGF21. More studies are required to understand how amino-acids metabolism is affected by treatment with glucagon/GLP-1 receptor co-agonists and FGF21 analogues in humans. Interestingly, both GLP-1/glucagon receptor co-agonists [566, 567] and FGF21 analogues [194, 564] have been shown to reverse NASH in preclinical models and both concepts are currently being tested in clinical development for this indication.

5 Part III-b: FGF19 and FGF21 – two sides of the same coin?

5.1 Introduction

The FGF19 gene was cloned in 1999 by homology to the mouse orthologue *Fgf15* from retina [43]. FGF19 is expressed in the ileal enterocytes [21] and released into the enterohepatic circulation in response to bile acids by activation of the FXR [45, 46]. FGF19 down regulates hepatic bile acid synthesis by inducing transcription of the SHP, which decreases the expression of the rate-limiting enzyme CYP7A1 in bile acid metabolism [44]. FGF21 is, as previously discussed, highly expressed in the liver and FGF21 regulates glucose, lipid and energy homeostasis in response to PR. FGF19 and FGF21 are, therefore, differently expressed and have separate physiological functions. Hence, it is of peculiar interest to understand why treatment with both rhFGF19 and rhFGF21 have metabolic benefits (BG and BW lowering) in mice [70]. FGF15 is the murine orthologue of FGF19 but FGF15 and FGF19 have low sequence homology. We therefore hypothesized that FGF15 does not mimic the BG lowering effect of FGF19 in mice. Interestingly, both FGF19 [568] and FGF21 [194] analogues are in clinical development for treatment for NASH and it is of great interest to understand if FGF19 and FGF21 also have overlapping effects in humans.

5.2 FGF19/15, FGF21 and FGF receptor homology

The homology between FGF19/FGF15, FGF21 and the relevant receptors across species is shown in Table 12. Mouse and human FGF21 share approximately 80% amino acid homology while mouse FGF15 and human FGF19 only share 52% amino acid identity. The FGFR binding domains (D2 and D3) are, as previously described, highly conserved between species, while the extracellular domains of KLB share approximately 80% of amino acids between human and mouse. The homology between human FGF19 and human FGF21 is 35% [569].

TABLE 12 FGF19 SUBFAMILY - LIGAND AND RECEPTOR HOMOLOGY ACROSS SPECIES

% identity to human	Human	Rhesus monkey	Rat	Mouse
FGF21	100	96	81	81
FGF19/FGF15	100	98-99	51	52
FGFR1c (D2/D3)	100	100	100*	100
FGFR4 D2/D3	100	100*	96	97
KLB extra-cellular)	100	97	82	79

The sequence alignment as well as % identity was calculated using AlignX, *=1 amino acid difference

5.3 Overlapping pharmacology of FGF19 and FGF21

FGF19 tg mice have reduced BW, increased EE, increased insulin sensitivity and do not develop hepatic steatosis [570]. Furthermore, FGF19 tg mice have decreased plasma IGF-1 [570]. Adenovirus-mediated (AAV) FGF19 delivery lowers BG and glycosylated HbA1c in *db/db* mice [571] and rhFGF19 treatment lowers BG and circulating bile acids in *db/db* mice [18](X). FGF19 has been described to induce hepatic glycogen synthesis, which may contribute to a BG lowering effect [572]. The metabolic consequences of FGF19 overexpression and treatment therefore resemble mice treated with rhFGF21 and FGF21 overexpressing tg mice. However, in contrast to FGF21 tg mice, which are protected towards HCC [227] FGF19 tg mice develop HCC [573, 574]. The mitogenic potential of FGF19 is mediated by activation of the FGFR4/KLB complex [575] which indirectly phosphorylates STAT3 inducing a proliferative effect [576]. FGF21 does not activate the FGF4/KLB complex even though binding has been observed [18, 33](X). High concentrations of FGF19 have furthermore, been shown to activate FGFR1c and FGFR4 in a heparin-dependent and KLB-independent manner [577] similar to signaling by the canonical FGFs [19]. FGFR4 antibodies are currently being investigated for treatment of HCC [578]. Antibodies towards FGF19 have also been suggested to target HHC but resulted in increased plasma bile acids and liver toxicity in monkeys [579] in agreement with lack of CYP7A1 suppression and large increases in plasma bile acids [580].

The FGF19 analogue NGM282 is in clinical development for the treatment of NASH. NGM282 is non-mitogenic and does not induce liver proliferation [581] but maintains the ability to suppress hepatic *Cyp7a1* gene expression in mice [568]. Furthermore, NGM282 decreases hepatic steatosis in mice [582] and in humans [568]; however, rhFGF19 increases total plasma cholesterol and TG in mice [18, 162](X) and plasma cholesterol in humans [568, 583]. The negative effect of FGF19 on plasma cholesterol is likely mediated by decreases in CYP7A1 [18, 162](X), while the adverse effect on plasma TG may be linked to a decrease in FXR activity (FXR KO mice have increase plasma TG) [584]. Oppositely, FGF21 and FGF21 analogues lower plasma TG and cholesterol in mice [6, 7] and humans [8, 194]. Nevertheless, FGF21 has also been shown to regulate bile acid synthesis in mice and monkeys [87], although much less potently than FGF19 [87] and more data are required to understand the impact of FGF21 treatment on bile acids in humans. Plasma FGF19 [572] and FGF21 [349] are increased postprandially in humans and both molecules have been suggested to increase postprandial insulin sensitivity [349, 572]. However, only FGF21 has been implicated in PR [60], while FGF19 been shown to increase hepatic protein synthesis in mice [572]. Thus, it is of interest to understand why both FGF19 [568] and FGF21 [194] analogues decrease hepatic steatosis and liver stiffness in humans.

5.4 Pharmacological action of FGF15 in mice

Mice lacking FGF15 have increased hepatic *Cyp7a1* mRNA expression and increased fecal bile acid excretion [44] showing overlapping effects with FGF19 [28]. Furthermore, genetic ablation of the *KLB* gene in mice increases bile acid synthesis and hepatic *Cyp7a1* expression level [49]. Adenovirus-mediated overexpression of FGF15 in mice decreases hepatic *Cyp7a1* and plasma bile acids [571, 585], but opposite to FGF19, AAV delivery of FGF15 does not lower BG in *db/db* mice [571]. However,

AAV delivery of FGF15 decreases BW and improves glucose tolerance in DIO mice after four weeks of treatment but with low efficacy compared to FGF19 [571]. FGF15 is, as FGF19, also implicated in development of HCC [586] but in mice FGF15 is less tumorigenic compared to FGF19 [571]. FGF15 has a unique free cysteine in position 135 (Cys110 in the mature protein), which is not found in other members of the FGF19 subfamily and FGF15 circulates as a dimer [571]. To overcome the difficulties in expressing and purifying FGF15 from E. coli, an FGF15 variant in where the surface cysteine at position 110 was mutated to serine (FGF15CS) was designed [18](X). Due to lack of native FGF15 protein, the potency of rmFGF15CS could not be compared to rmFGF15, but the variant was shown to be active *in vitro* and *in vivo* [18](X).

5.5 Receptor potency and selectivity in mice and human

It has for unknown reasons been difficult to develop classical ligand replacement binding assays for FGF21. Therefore, immunoprecipitation [27], protein-protein interaction (surface plasmon resonance (SPR) technology) [86, 575] and cell-based assays overexpressing FGFR and KLB [27] have been used to determine potency and receptor selectivity of FGF19 and FGF21. In cellular systems FGF19 and FGF21 have overlapping receptor selectivity and bind FGFR1c, FGF2c, FGFR3c, and FGFR4 in the presence of KLB [31]; however, as mentioned above only FGF19 signals through the FGFR4/KLB complex [31, 34, 587]. FGF21 has a weak affinity towards the FGFR2c/KLB complex [33]. The pharmacological effects of FGF21 are mediated by activation of specific regions of the CNS and the adipose tissue, which expresses KLB and the short isoforms of FGFR1 and FGFR3 [91, 588]. The FGFR4/KLB receptor complex is highly expressed in the hepatocytes and is required for FGF15/FGF19 to regulate bile acids synthesis and the FGFR4 KO mice [34] and FGF15 KO mice [238] have overlapping phenotypes with high serum bile acid and low plasma TG. Additionally, an FGFR1c selective FGF19 analogue (FGF19v) (1-20 FGF21 and 25-194 FGF19) does not induce proliferation or decrease bile acids in mice [34], while the FGF19v analogue is able to lower BG in *ob/ob* mice [34]. Using the FGF19v variant, it was shown that the receptor selectivity of FGF19 and FGF21 is mediated via the N-terminal part of the molecule [186] while the C-terminal of FGF19 and FGF21 bind to KLB. Recent studies have, however, shown that specific changes in the C-terminal of FGF21 also affect the receptor selectivity [589].

The Alpha SureFire technology is based on generating a signal by bringing acceptor and donor beads in proximity to one another and was used to develop an FGF21 binding assay using biotinylated FGF21 (122C-biotinylated FGF21) [18](X). A biotinylated FGF21 can be coupled to streptavidin donor beads and an Fc-fusion ectodomains of the various FGFR can be coupled to the protein A acceptor beads. Addition of KLB protein to the assay brings the donor and acceptor beads in close proximity and induces a light signal. Therefore, addition of recombinant FGFs proteins will replace the biotinylated FGF21 and decrease the signal [18](X). Based on the concentration required to replace half of the biotinylated FGF21 a binding affinity can be estimated [18](X). Using this binding assay, it became clear that rmFGF15CS cannot bind human KLB, but was able to bind FGFR1c and FGFR4 in the presence of mouse KLB with similar binding affinities. Oppositely, rhFGF19 and rhFGF21 bind the FGFR1c and FGFR4 in the presence of both murine and human KLB. Recombinant hFGF21 binds with higher affinity to the FGFR1c/KLB (mice and human) complex than to the FGFR4/KLB complex in agreement with other reports [587]. In the presence of human KLB, rhFGF19 binds the FGFR4 with 18-fold higher affinity than FGFR1c, while rhFGF19 in the presence of mouse KLB binds FGFR1c/KLB and FGFR4/KLB with similar affinities [18](X). Therefore, rmFGF15CS and rhFGF19 displayed similar receptor selectivity in the presence of mouse KLB. Surprisingly, compared to rhFGF19, which increases glucose uptake in mouse adipocytes, no effect of rmFGF15CS was observed. As rmFGF15CS binds to the murine FGFGR1c/KLB, this was unexpected. Subsequent experiments showed that

rmFGF15CS is unable to induce downstream signaling in HEK293 (primarily expressing FGFR1c) transfected with mouse KLB [18](X). These data may therefore explain why rmFGF15CS was unable to lower BG in *db/db* mice [18, 571](X). Conversely, both rhFGF19 and rmFGF15SC inhibit *Cyp7A1* mRNA expression at low nM concentrations in primary rat hepatocytes while higher concentrations of rhFGF21 are required [18](X). Furthermore, FGF21 is approximately 1000-fold less potent than FGF19 in inhibiting *CYP7A1* mRNA expression in primary human hepatocytes [87] in agreement with binding data [18](X). In *db/db* mice, rhFGF19 and rmFGF15SC increased plasma TG and cholesterol as previous published for FGF19 [162]. The effect of FGF15 and FGF19 in the CNS is currently not understood but endogenous FGF15 which circulates as an dimer [571] may not cross the BBB as described for FGF21 [177]. However, hypothalamic FGF15 has been described to regulate glucagon secretion [590].

The receptor selectivity and pharmacological effects of rmFGF15CS, rhFGF19 and rhFGF21 in binding and cellular assays as well as in mice are summarized in Figure 15. In contrast to rmFGF15CS, rhFGF19 signals through FGFR1c/KLB and induces an anti-diabetic effect in mice. This support the hypothesis that FGF15 is not mimicking all FGF19 effects in mice. Therefore, data where rhFGF19 has been used as a surrogate protein for FGF15 in mice, should be interpreted with care, as the two orthologues display differential receptor selectivity. Moreover, as rhFGF19 can signal through the FGFR1c and human KLB receptor complex [589] rhFGF19 and rhFGF21 must have overlapping effects in humans.

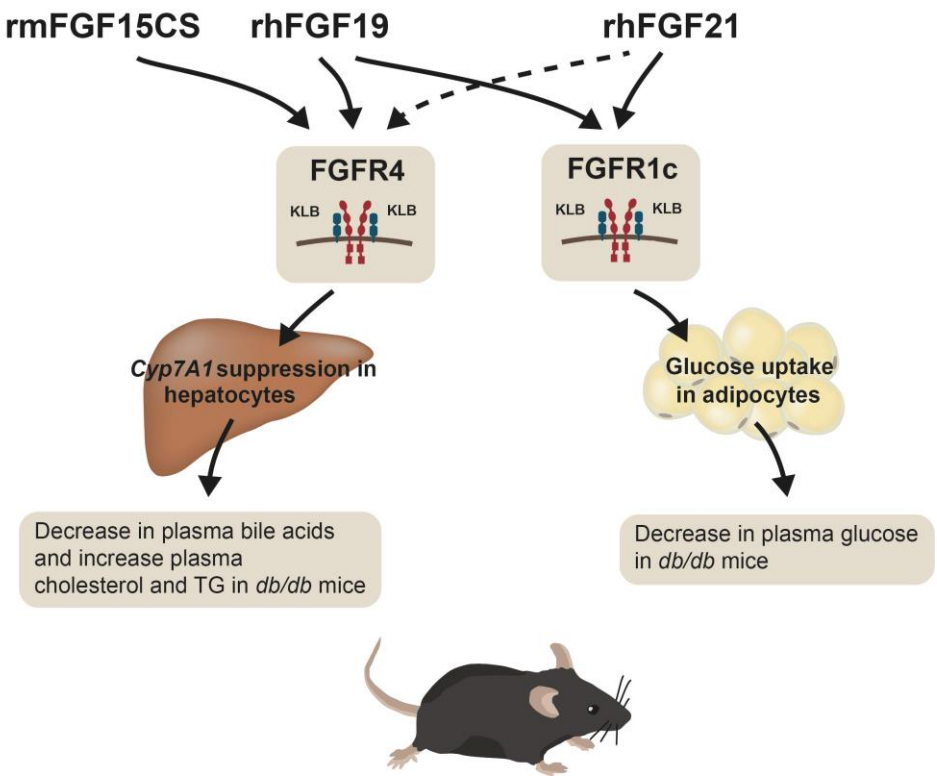

FIGURE 15 FIGURE 14 DIFFERENTIAL EFFECTS ON FGF15, FGF19 AND FGF21 (DATA FROM PUBLICATION X)

recombinant hFGF19 and the rmFGF15CS bind and signal through the FGFR4/KLB complex and regulate bile acids in vitro and in vivo. Recombinant hFGF19 and rhFGF21 bind and signal through the FGFR1c/KLB complex and regulate glucose metabolism in vitro and in vivo.

5.6 FGF19 and FGF21 analogues for the treatment of NASH

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disorder in Western countries and in the USA 30% of the adult population suffers from NAFLD [591]. Simple steatosis can if not treated progress to NASH, which is defined by the presence of steatosis, lobular inflammation, portal hypertension, cellular ballooning and varying degrees of fibrosis [592]. Eventually, NASH can lead to cirrhosis and HCC [593]. Obesity-induced NASH and liver cirrhosis represent the third most common cause of liver transplantation in the USA and is expected to be the primary cause of liver transplantation by 2030 [594]. Furthermore, patients with NASH have an overall higher mortality rate compared to age-matched controls and the primary cause of death in the early stages of NASH is cardiovascular disease, while the cause of death in patients with late stage fibrosis is liver related [595]. There is currently no approved treatment for NASH.

5.6.1 NGM282

The non-mitogenic FGF19 analogue NGM282 described above has been studied in several clinical trials. In healthy volunteers, NGM282 decreased serum levels of 7α-hydroxy-4-cholesteb-3-one (C4) [596] and showed target engagement by inhibition of hepatic CYP7A1. In a phase 2 trial in patients with primary biliary cholangitis, a devastating liver disease caused by hepatic accumulation of toxic bile acids [597], administration of NGM282 for 28 days lowered plasma bile acids and improved liver function [598]. NGM282 is in general well tolerated, but dosing of NGM282 is associated with dose-related abdominal cramping and diarrhea. NGM282 was in a follow-up study shown to alter bowel function and accelerates gastric and colonic transit [599]. The most commonly observed side effects of NGM282 were injection side reactions and GI side effects (diarrhea, abdominal pain and nausea) while no difference in fecal fat or weight was observed [599]. Up to 14% of subjects in the high doses reported increases in appetite [599]. Furthermore, in a phase 2 study in patients with NASH 12 weeks of NGM282 treatment reduced absolute liver fat by 5% measured by magnetic resonance imaging proton density fat fraction (MRI-PDFF) in 80 % of the patients [568]. A significant decrease in plasma liver enzymes alanine aminotransferase (ALT) and aspartate amino transferase (AST) was also observed in response to NGM282 treatment [568]. Furthermore, plasma C4 was decreased by more than 95% within the first day of treatment and a significant increase in plasma LDLc was observed while plasma TG was decreased [568]. A significant decrease in BW was observed in the highest dose group [568]. Co-administration with statins was, however, able to normalize the NGM282-induced increases in plasma LDLc [583]. However, as the primary cause of death in patients in the early stages of NASH is caused by cardiovascular events, increases in LDLc may challenge the development of FGF19 analogues for the treatment of NASH [595].

5.6.2 Pegbelfermin

Several FGF21 analogues have been tested in humans as described previously [8, 9]. All FGF21 analogues have been shown to lower lipids (TG and LDLc), while varying effects on BG and BW have been observed. Pegbelfermin, a PEGylated FGF21 analogue, is currently in development for NASH. In a Phase 2 clinical trial 16 weeks of pegbelfermin treatment decreased absolute hepatic lipid content by 6.8% measured by MRI-PDFF [194]. Furthermore, pegbelfermin increased plasma adiponectin and HDLc while fasting plasma LDLc and TG was decreased. Liver stiffness, measured by magnetic resonance elastography (MRE), was also decreased, as well as a biomarker of fibrogenesis (N-terminal fragment of type III collagen (ProC3)) [600] [194]. A summary of the pharmacological effects on FGF21 and FGF19 analogues in humans are shown in Table 13, highlighting that both FGF19 and FGF21 analogues lower hepatic steatosis and fibrotic biomarkers in humans. However, differential effects on plasma cholesterol and TG are observed. Future clinical studies are required to determine which approach is more beneficial for patients with NASH and if blockage of bile acid synthesis,

which may increase plasma LDLc and bowel movement, is advantageous and acceptable. It is of interest to observe that NGM282 increases appetite, like reported for several of the FGF21 analogues [9, 201, 204], thus overlapping effects on regulation of appetite and food preferences may appear. The effect of FGF21 on bile acids in humans has not been determined while studies in monkeys show that FGF21 also decreases plasma bile acids [87].

TABLE 13 PHARMACOLOGICAL EFFECTS OF FGF19 AND FGF21 ANALOGUES IN HUMAN

Compound	**NGM282** [568, 583, 598, 601]	**Pegbelfermin** [10, 194]
BG	↔	↔
BW	↔	↔
Insulin sensitivity	↔	↑
Hepatic steatosis	↓	↓
Hepatic fibrosis	↓	↓
Serum Pro-C3	↓	↓
Plasma TG	↓	↓
Plasma cholesterol	↑	↓
Serum C4	↓	ND
Plasma adiponectin	ND	↑

ND: not determined.

5.7 Conclusion

The FGF19 story is classical example of misinterpretation of mouse data. The low homology between FGF15 and FGF19 should have caused caution when the rhFGF19 was used to understand effect of FGF15 in mice. The mouse orthologue does not activate the FGFR1c/KLB complex and only regulates bile acids metabolism in mice, in contrast to rhFGF19 which like rhFGF21 decreases BW and BG. Therefore, the hypothesis that FGF19 cannot be used as a FGF15 surrogate in mice is substantiated. However, in humans FGF19 and FGF21 analogues are likely to have overlapping metabolic effects on steatosis and fibrosis, but while FGF21 analogues lower plasma lipids, NGM282 has been shown to increase plasma cholesterol, highlighting major difference in the regulation of bile acid metabolism between FGF19 and FGF21 in humans.

6 Dansk resumé

Prækliniske dyremodeller er nødvendige for lægemiddeludvikling. Valg af dyremodellerne er vigtig når et muligt lægemiddel skal undersøges for effekter og bivirkninger før klinisk testning. Homologi af aminosyresekvenser af det protein, som skal undersøges, og dets receptorer på tværs of arter samt forståelse af de farmakokinetiske egenskaber er vigtige faktorer, som kan påvirke, hvordan et stof virker i prækliniske dyremodeller. På trods af, at mange af disse faktorer har været tilstede, har det været en stor udfordring at forstå hvorfor, den imponerende effekt af fibroblast growth factor 21 (FGF21) på vægttab og sukkersyge observeret i overvægtige og diabetiske mus, grise og aber, ikke er observeret i de kliniske studier, der har været udført på mennesker med analoger af FGF21. Dog ved man, at FGF21 har metaboliske effekter i mennesker og efter dosering ses en sænkning of plasma triglycerider og plasma kolesterol, hvilket også er set i flere prækliniske modeller.

FGF21 er en del af FGF19 subfamilien, som er kendetegnet ved at have metaboliske effekter. FGF21 binder til den korte isoform of FGF receptor 1 and 3, men kun i tilstedeværelse af ko-receptoren beta-klotho (KLB). FGF21 receptorkomplekset er udtrykt i specifikke områder af hjernen samt i fedtvævet. FGF21 har pleiotropiske metaboliske effekter på energi- og substrat-metabolismen og behandling med FGF21 normaliserer blodglukosen i gnavermodeller med Type 1 og Type 2 diabetes,

samt i diabetiske aber. Herudover nedsætter FGF21 plasma insulin og øger insulinfølsomheden i overvægtige ikke diabetiske aber. Behandling med FGF21 nedsætter også kropsvægten ved at øge energiomsætningen i højfedtfodrende mus, rotter og aber, mens FGF21 ser ud til at nedsætte fødeindtagelsen i chowfodrende grise og aber. Der ses også et fald i plasma triglycerider og kolesterol i FGF21 behandlede dyr. Baseret på disse resultater i prækliniske modeller ser FGF21 ud til at være en potentiel unik lægemiddelkandidat med effekter på både kropsvægt, sukkersyge samt dyslipidæmi.

Disse positive resultater initierede et kapløb om at identificere en FGF21 analog, som kunne anvendes til humant brug. I de første kliniske studier i overvægtige type 2 diabetikere sås en imponerende effekt på plasma lipider, mens FGF21s evne til at sænke blodsukkeret og kropsvægten var mindre overbevisende. Dog sås et fald i faste plasma insulin. Disse observationer er i stærk kontrast til de positive effekter, som man havde observereret i dyr. Der er muligvis flere årsager til, at FGF21s effekter i dyr ikke translaterer til mennesker, men den fysiologiske rolle af FGF21 må tages i betragtning. FGF21 regulerer energiindtag og forbrænding i respons til en lav protein diæt. FGF21-behandling påvirker derfor potentielt appetit og forbrænding afhængigt af protein og fedtindhold i de diæterne, der anvendes i de prækliniske dyreforsøg og dette bliver ikke kontrolleret på samme måde i de humane studier. Dette er påvirker muligvis den anti-diabetiske og kropsvægtssænkende effekt af FGF21 behandling i mennesker. I slanke mus, der overudtrykker FGF21, ses en nedsat fertilitet i hun-mus, og nedsat knoglemineralisering og forhøjet plasma kortikosterone i både hun- og han-mus. Disse fund kunne være relateret til mangel på energi og, af stor vigtighed, ses ingen ændring i knoglemineralisering ellers plasma kortisol i overvægtige grise og aber behandlet med FGF21.

Mens den farmakologiske effekt af FGF21 var veletableret i prækliniske modeller var FGFs fysiologiske rolle ikke velbeskrevet. FGF21 var oprindeligt beskrevet til at være involveret i regulering af de metaboliske ændringer i respons til faste, da plasma FGF21 var øget efter en kort faste i mus. Dog er plasma FGF21 også øget i overvægtige mus, aber og mennesker, hvilket indikerer en kompleks regulering af plasma FGF21. Herudover øger FGF21 behandling forbrændingen og insulinfølsomheden i præ-kliniske modeller, hvilket er i kontrast til det metaboliske respons til faste, som involverer nedsat forbrænding og nedsat insulinfølsomhed. Derfor var der ikke en klar sammenhæng mellem behandlingseffekter og den fysiologiske rolle af FGF21. Herudover øger 2-3 dages faste i mennesker ikke plasma FGF21, men en signifikant nedregulering af FGF21 receptorkomplekset ses i fedtvævet. Dette indikerer, at FGF21-aktiviteten er nedsat under faste i mennesker. Plasma FGF21 er på den anden siden øget i respons til en oral glukosetolerancetest og i respons til en lavproteindiæt i mennesker, hvilket demonstrer, at sammensætning af kosten har stor betydning for regulering af plasma FGF21. FGF21 spiller altså en vigtig rolle i de metaboliske forandringer, som sker i forbindelse med indtag et af en diæt, som mangler proteiner både akut og kronisk. Den metaboliske adoption til mangel på protein involverer ændring i appetit, fødevarer-præferencer og forbrænding, samt øget glukose- og fedt-optagelse i fedtvævet. Herudover, regulerer FGF21 muligvis væksthormon/IGF-1-aksen for at øge insulinfølsomheden.

Den komplekse FGF21s biologi har også gjort det svært at skelne mellem effekter af FGF21 og glukagon, samt FGF21 og FGF19. Plasma FGF21 øges i respons til en glukagon-injektion i mus og mennesker, men plasma FGF21 er højere i mus, der mangler glukagon receptoren (Gcgr knockout (KO) mus). Herudover, er den anti-diabetiske effekt, observeret i diabetiske Gcgr KO, svækket i mus, hvor plasma FGF21 er neutraliseret. FGF19 udskilles fra tarmceller i respons til høje galdesyrekoncentrationer og via FGF19 receptorer i hepatocytter regulerer FGF19 galdesyredannelse. Selvom FGF19 og FGF21 har helt forskellige fysiologiske effekter, sænker

behandling med både FGF19 og FGF21 blodglukosen i diabetiske mus. Det er dog vigtigt at bemærke, at FGF19s orthlogue FGF15 kun regulere galdesyredannelsen og ikke sænker blodglukosen i diabetiske mus, hvilket støtter hypotese om, at FGF19 ikke kan bruges som et surrogat for FGF15 i mus.

Det kan derfor konkluderes, at den øgede forståelse af FGF21's fysiologiske rolle har gjort det lettere at forklare de farmakologiske effekter af FGF21. Dette forklarer muligvis også, hvorfor effekter af FGF21 observeret i præ-kliniske arter ikke translaterer til mennesker, da FGF21 sandsynligvis påvirker appetitten, fødepræferencer og forbrændingen i både de prækliniske og kliniske studier, men hvor diæten i de prækliniske studier er nøje defineret er dette ikke fastsat i de kliniske studier. De bivirkninger, der er observeret i stanke mus, er sandsynligvis ikke relevante for overvægtige patienter med T2D eller non-alkoholisk fedtlever, men yderligere studier er nødvendige for at etablere mulige behandlingseffekter i mennesker sammenholdt med de mulige bivirkninger. FGF21 analoger er i udvikling for behandling af non-alkoholisk fedtlever og i både mus og mennesker, er der er set positive effekter på steatose og fibrose efter behandling med FGF21. FGF21 kan derfor muligvis bruges til behandling af pateinter med non-alkoholisk fedtlever, hvilket er en sygdom med høj dødelighed og hvor der ikke er nogen godkendt behandling.

7 List of Abbreviations

AAV: adeno-associated virus

Ab: antibody

ACTH: adrenocorticotropic hormone

Agrp: agouti-related protein

AGTL: adipose triglyceride lipase

ALT: alanine aminotransferase

AMPK: AMP-activated protein kinase

AN: anorexia nervosa

Ang: angiotensin

AP: area postrema

AST: aspartate aminotransferase

ATF4: activating transcription factor 4

β3-AR: β-3-adrenergic

BAT: brown adipose tissue

BBB: blood brain barrier

BCAA; branched chain amino acids

BG: blood glucose

BHB: β-hydroxybutyrate

BMD: bone mineral density

BMI: body mass index

BP: blood pressure

BSAP: bone specific alkaline phosphatase

BT: body temperature

BW: body weight

C4: 7α-hydroxy-4-cholesteb-3-one

Camk2; calcium/calmodulin-dependent kinase II a α

CCK: cholecystokinin

CCL11: C-C motif chemokine 11

CD36: cluster of differentiation 36

ChREBP: Carbohydrate-responsive element-binding protein

CNS: central nerve system

CRF: corticotropin releasing factor

CRP: C-reactive protein

CSF: cerebrospinal fluid

Ct: cycle threshold

CTX: carboxy-terminal collagen crosslinks

CVD: cardiovascular disease

Cyp7a1: cholesterol 7 alpha-hydroxylase

D2: domain 2

D3: domain 3

DAG: diacylglycerol

db/db: mouse with mutation in the gene encoding the leptin receptor

DIO: diet-induced obese

DNL: de novo lipogenesis

EE: energy expenditure

ELISA; enzyme-linked immunosorbent assay

EMC; extracellular matrix

FAP: fibroblast activating protein

FFA: free fatty acids

FGF: Fibroblast growth factor

FGFBP3; FGF binding protein 3

FGFR; fibroblast growth factor receptor

FGR: early growth response

FI: food intake

FRS; FGFR substrate

FXR: farnesoid X receptor

Gcgr: glucagon receptor

GLP-1: Glucagon-like peptide 1

GCN2: general control nonderepressible 2

GFP: green florescent protein

GH: growth hormone

GI: gastrointestinal

GLP-1: glucagon-like peptide-1

GLUT: glucose transporter

GnRH: gonadotropin releasing hormone

GPAT1: glycerol-3-phosphate acyltransferase 1

GRB2: Growth Factor Receptor-Bound protein 2

GSH: glutathione

GWAS: genome-wide association studies

HbA1c: glycated haemoglobin

HCC: hepatocellular carcinomas

HDLc: High-density lipoprotein cholesterol

HPA: hypothalamic-pituitary-adrenal

HPG: hypothalamic–pituitary–gonadal

HS: heparan sulfate

HSL: hormone sensitive lipase

ICV: intracerebroventricular

IGF-1: Insulin like growth factor 1

IGFBP: Insulin like growth factor binding protein

IL: interleukin

IP: intraperitoneal

IV: intravenous

IVGTT: Intra-venous glucose tolerance test

KD: ketogenic diet

KL: alfa-klotho

KLB: beta-klotho

km: Michaelis-Menten constant

KO: knockout

LDLc: low-density lipoprotein cholesterol

LH: luteinizing hormone

LPL'ase: lipoprotein lipase

MAPK: mitogenic-activated protein kinase

ME: media eminence

MRE: magnetic resonance elastography

MRI-PDFF: magnetic resonance imaging proton density fat fraction

mRNA: messenger RNA

mTOR: mammalian target of Rapamycin

Mw: molecular weight

NAFLD: non-alcoholic fatty liver disease

NASH: non-Alcoholic Steatohepatitis

ND: not determined

NHP: non-human primate

NKT: natural killer T

NPY: neuropeptide Y

NTS: solitary tract

Ob/ob: mouse with mutation in the gene encoding leptin

OGTT: oral glucose tolerance test

OVX: ovariectomized

P1NP: N-terminal pro-peptide of type I procollagen

PCSK9: proprotein convertase subtilisin/kexin type 9

PD: pharmacodymanics

Pepck : phosphoenolpyruvate carboxykinase

PGC1: peroxisome proliferator-activated receptor gamma coactivator 1

PI3K: phosphoinositide 3-kinase

PK: pharmacokinetics

PPAR: peroxisome proliferator-activated receptor

PR: protein restriction

ProC3: N-terminal fragment of type III collagen

PVN: paraventricular nucleus

PYY: peptide YY

RA: rheumatic arthritis

Reb-Erv: NR1D1 (nuclear receptor subfamily 1, group D, member 1)

rh: recombinant human

rm: recombinant mouse

Rq: respiratory quotient

RT: room temperature

RYGB: roux-en-Y gastric bypass

sc: subcutaneous

SCN: suprachiasmatic nuclei

SGLT2: sodium glucose co-transporter 2

SHP: small heterodimer partner

Sirt1: NAD-dependent deacetylase sirtuin-1

SNP: single nucleotide polymorphism

SNS: sympathetic nerve system

SOD: super oxide dismutase

SREBP: sterol regulatory element-binding protein

SPR: surface plasmon resonance

STAT3: signal transducer and activator of transcription 3

STZ: streptozotocin

T½: half-life

T1D: type 1 diabetes

T2D: type 2 diabetes

T3: triiodothyronine

T4: thyroxine

TBA: total bile acids

tg: transgenic

TG: triglycerides

TGR5: G-protein-coupled bile acid receptor

TNF: tumor necrosis factor

UCP: uncoupling protein

VLCD: very low-calorie diet

VLDL: very low-density lipoproteins

WAT: white adipose tissue

wt: wildtype

ZDF: Zucker Diabetic Fatty

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