Trends in Pharmacological Sciences

# TARGETING FGF21 FOR THE TREATMENT OF NASH

--Manuscript Draft--

| | |
|---|---|
| **Manuscript Number:** | TIPS-D-19-00189R1 |
| **Article Type:** | Review |
| **Keywords:** | FGF21; NASH; fibrosis; NAFLD; steatosis; triglyceride; inflammation. |
| **Corresponding Author:** | Manuel Vazquez-Carrera<br><br>Barcelona, SPAIN |
| **First Author:** | Mohammad Zarei |
| **Order of Authors:** | Mohammad Zarei |
| | Javier Pizarro-Delgado |
| | Emma Barroso |
| | Xavier Palomer |
| | Manuel Vázquez-Carrera |
| | Manuel Vazquez-Carrera |
| **Abstract:** | Nonalcoholic steatohepatitis (NASH), the severe stage of nonalcoholic fatty liver disease (NAFLD), is defined as the presence of hepatic steatosis with inflammation and hepatocyte injury and different degrees of fibrosis. Although NASH affects 2-5% of the global population, no drug has been specifically approved to treat the disease. Fibroblast growth factor 21 (FGF21) and its analogs have emerged as a potential new therapeutic strategy for the treatment of NASH. In fact, FGF21 deficiency favors the development of steatosis, inflammation, hepatocyte damage and fibrosis in the liver, whereas administration of FGF21 analogs ameliorates NASH by attenuating these processes. Here, we review mechanistic insights into the beneficial and potential side effects of therapeutic approaches that target FGF21 for the treatment of NASH. |

UNIVERSITAT DE BARCELONA

Manuel Vázquez Carrera
Department of Pharmacology
Faculty of Pharmacy
Diagonal 643
Barcelona E-08028
SPAIN
Phone: 34 93 4024531
Fax: 34 93 4035982
E-mail: mvazquezcarrera@ub.edu

December 9th, 2019

Dr. Kusumika Mukherjee
Editor, *Trends in Pharmacological Sciences*

**TIPS-D-19-00189**

Dear Dr. Mukherjee,

According to your instructions we wish to submit a reworked version of our manuscript entitled "Targeting FGF21 for the treatment of NASH".

We submit a letter where we have addressed, point-by-point, all the issues raised by the reviewers. I thank you in advance for your interest in our work.

Yours truly,

Manuel Vázquez-Carrera

## Highlights

No drug has yet been approved specifically for treating nonalcoholic steatohepatitis (NASH), the necroinflammatory form of nonalcoholic fatty liver disease (NAFLD) which confers a higher risk of progression to advanced fibrosis, cirrhosis and hepatocellular carcinoma.

Targeting fibroblast growth factor 21 (FGF21), a hormone with insulin-sensitizing and hepatoprotective properties, has emerged as an option for NASH therapy.

FGF21 analogs have demonstrated their efficacy in both animal models and humans with NASH, although some concerns have been raised about the safety of FGF21 analogs in humans.

Strategies other than the use of FGF21 analogs that potentiate the effects of FGF21 and might be useful in the treatment of NASH are also being developed.

# TARGETING FGF21 FOR THE TREATMENT OF NASH

*Mohammad Zarei*, Javier Pizarro-Delgado, Emma Barroso, Xavier Palomer, Manuel Vázquez-Carrera*

*Department of Pharmacology, Toxicology and Therapeutic Chemistry, Faculty of Pharmacy and Food Sciences, University of Barcelona; Institute of Biomedicine of the University of Barcelona (IBUB); Pediatric Research Institute-Hospital Sant Joan de Déu; and the Spanish Biomedical Research Centre in Diabetes and Associated Metabolic Diseases (CIBERDEM)-Instituto de Salud Carlos III, Avinguda Joan XXII 27-31, E-08028 Barcelona, Spain*

***Correspondence:*** mvazquezcarrera@ub.edu (M. Vázquez-Carrera)

*****Presente address: Center for Mitochondrial and Epigenomic Medicine and Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia and Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104.

**Keywords:** FGF21; NASH; fibrosis; NAFLD; steatosis; triglyceride; inflammation.

**Abstract**

Nonalcoholic steatohepatitis (NASH), the severe stage of nonalcoholic fatty liver disease (NAFLD), is defined as the presence of hepatic steatosis with inflammation and hepatocyte injury and different degrees of fibrosis. Although NASH affects 2-5% of the global population, no drug has been specifically approved to treat the disease. Fibroblast growth factor 21 (FGF21) and its analogs have emerged as a potential new therapeutic strategy for the treatment of NASH. In fact, FGF21 deficiency favors the development of steatosis, inflammation, hepatocyte damage and fibrosis in the liver, whereas administration of FGF21 analogs ameliorates NASH by attenuating these processes. Here, we review mechanistic insights into the beneficial and potential side effects of therapeutic approaches that target FGF21 for the treatment of NASH.

## NAFLD/NASH: a burgeoning health problem

Nonalcoholic fatty liver disease (NAFLD), the most common form of liver disease, is a pathological entity that ranges from isolated steatosis or NAFL (defined as the presence of cytoplasmic triglyceride [TG] droplets in more than 5% of hepatocytes with no evidence of hepatocellular injury) to nonalcoholic steatohepatitis (NASH). The latter is a more advanced stage that is distinguished from NAFL by the presence of inflammation, hepatocyte damage (hepatocyte **ballooning** and cell death) and different degrees of collagen deposition (**fibrosis**) (see Glossary) [1]. These abnormalities develop in the absence of excessive alcohol intake (<20 g/day in women and <30 g/day in men) [2] and are strongly associated with obesity, insulin resistance (IR), type 2 diabetes mellitus (T2DM) and dyslipidemia [3]. The presence of NASH confers a higher risk of progression to advanced fibrosis, cirrhosis and hepatocellular carcinoma [4] and, in fact, NASH is expected to become the leading cause of liver transplants by 2020 [5]. In addition, NASH patients are at higher risk of cardiovascular disease, the major cause of morbidity and mortality among these patients. In line with the obesity epidemic, the global prevalence of NAFLD has risen in recent years and currently stands at 25%, whereas the global prevalence of NASH ranges from 2-5% [5,6].

Although considerable progress has been made in recent years in understanding the potential pathological mechanisms that underlie the development of TG deposition in the liver and the transition from NAFL to NASH and identifying many potential therapeutic targets, its treatment remains an unmet clinical need. In addition, the multiple mechanisms involved in the development of NASH make it likely that the treatment of this disease will require a single drug with diverse cellular/molecular targets or the use of combination therapies.

## Pathophysiological mechanisms in NASH: the basics

NASH is a very complex disease whose development involves many pathological drivers with different contributions in each patient. As a result, a high heterogeneity is observed in the molecular mechanisms and clinical manifestations of the disease [7], and some patients may even develop NASH from the beginning of disease, thus demonstrating that isolated steatosis does not always precede NASH.

Although the two-hit hypothesis was initially proposed to explain the development of NASH, it has now been substituted by the multiple-hit hypothesis that implies the presence of different insults acting together and synergistically on genetically predisposed subjects to induce this condition. In the presence of the IR and dysregulated lipid metabolism observed in obese patients, isolated steatosis develops and renders hepatocytes more susceptible to multiple hits, including mitochondrial dysfunction, oxidative damage, adipokine imbalance, dysregulated apoptosis, activation of pro-inflammatory mediators and pro-fibrogenic factors, hepatic stellate cell activation and production of gut-derived toxins by microbiota (**Figure 1**). Liver steatosis is likely to be the first stage in the development of NAFLD and NASH. It results from a permanent imbalance between fatty acid (FA) influx and utilization and very low-density lipoprotein (VLDL) secretion. This imbalance may arise from: 1) Increased lipolysis of adipose tissue due to the presence of obesity-induced IR, thus provoking a higher uptake of FA by hepatocytes that will be used to synthesize TG; 2) Overnutrition with a higher consumption of fats or simple sugars (such as fructose), which are converted to TG in the liver through the *de novo* lipogenesis (DNL) process and also result in liver fat deposition. The DNL process is controlled by two transcription factors: sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate response element-

binding protein (ChREBP), which are activated by hyperinsulinemia and an excessive intake of simple sugars, respectively; 3) Impaired mitochondrial FA oxidation; 4) Increased secretion of VLDL that causes atherogenic dyslipidemia but does not compensate for the increased synthesis of TG.

Accumulation of inert TG in the liver has been considered as an adaptive response to an excess supply of FA that protects hepatocytes from the lipotoxic effects of surplus toxic free fatty acids (FFA) or from the synthesis of FA-derived lipotoxic species, such as ceramide, diacylglycerol (DAG) and lysophosphatidylcholine [8-10]. Indeed, in NAFL patients who develop NASH, the factor that determines the transition to NASH is likely the inability of their hepatocytes to cope with an overload of FA, which ultimately leads to liver injury. Once the physiologically adaptive mechanisms have been overcome, the excessive FA provokes a series of harmful consequences, known as lipotoxicity, leading to mitochondrial dysfunction, reactive oxygen species (ROS) generation, endoplasmic reticulum (ER) stress, activation of inflammatory pathways, hepatocellular injury and cell death. These changes lead to fibrosis and genomic instability, which predispose to cirrhosis and hepatocellular carcinoma. In addition to involve hepatocytes, NASH development is also associated with the activation of additional liver cells by lipid peroxidation products and other stimuli. Thus, the inflammatory response is exacerbated by the activation of **Kupffer cells**, the resident macrophages in the liver that also contribute to the activation of fibrosis. In fact, injured hepatocytes and activated Kupffer cells stimulate **hepatic stellate cells**, which are key in the development of fibrosis. This is one of the liver's responses to injury and stimulates the accumulation of extracellular matrix and may cause scar formation composed mainly of type 1 collagen, eventually leading to advanced fibrosis, cirrhosis and liver failure [11].

Mechanisms other than lipotoxicity play a role in the development of NASH, including genetic factors, dysfunctional gut microbiota, increased free cholesterol accumulation and adipokine imbalance. The genetic variant most strongly associated with an increased risk of NASH is a single-nucleotide polymorphism in the PNPLA3 (Patatin-like phospholipase domain-containing protein 3) gene that regulates hepatic TG lipolysis [12,13]. Changes in the microbiota caused by obesity or the consumption of high-fat diets (HFD) can lead to intestinal dysbiosis (defined as an imbalance between protective and harmful bacteria) and increased gut permeability, thus allowing bacterial endotoxins to reach the liver through the portal vein. Once in the liver, bacterial products such as lipopolysaccharide (LPS) activate membrane-bound Toll-like receptors (TLRs), which trigger the production of pro-inflammatory cytokines, thereby promoting the recruitment of immune cells and stimulating the inflammatory response, liver damage and fibrogenesis [14]. Excess dietary intake or perturbed cholesterol homeostasis can result in the accumulation of free cholesterol in the liver, which promotes apoptosis and necrosis in hepatocytes and contributes to inflammation in Kupffer cells and fibrogenesis in hepatic stellate cells [15]. The adipokine imbalance observed during adipose tissue associated to obesity contributes to the development of NASH [16]. One of the best examples is adiponectin, whose circulating blood levels are markedly reduced in visceral obesity and states of IR, such as NASH and T2DM. Adiponectin reduces steatosis in hepatocytes through the activation of FA oxidation and the reduction of FA influx and DNL. It also inhibits hepatocyte apoptosis, exerts anti-inflammatory effects and attenuates fibrosis by reducing the activation and proliferation of hepatic stellate cells, while inducing their apoptosis [17].

Hepatokines have recently emerged as potent regulators of NASH development. Among them, **fibroblast growth factor 21** (**FGF21**) **and its analogs** show promise as a potential therapeutic strategy for the treatment of NASH. This review highlights the current understanding of the actions of FGF21 on NASH and of how pharmacological therapies based on FGF21 are potential drugs for the future treatment of this disease.

## Effects of FGF21 in NASH

FGF21 is an atypical member of the complex endocrine FGF family without mitogenic activity and functions as a hormone with pleiotropic effects on glucose and lipid metabolism, overall resulting in insulin-sensitizing and hepatoprotective properties. FGF21 is expressed mainly in the liver [18], and circulating FGF21 derives largely from this organ and shows good correlation with the hepatic expression of FGF21 [19]. FGF21 is also expressed in brown adipose tissue [20], white adipose tissue and the acinar pancreas [21]. In addition to being released into the circulation, FGF21 may also act in an autocrine/paracrine manner. Skeletal muscle expresses very low levels of FGF21. However, under conditions of mitochondrial stress, such as those observed in muscle myopathies, skeletal muscle also releases FGF21 into the circulation [22]. In fact, FGF21 is considered to be a stress-induced hormone whose levels rise in metabolically compromised states, thereby suggesting that it could be useful as a marker for metabolic pathologies. Thus, elevated plasma FGF21 levels have been reported in a variety of pathologies, such as obesity [23], T2DM [24] and NAFLD [25]. The increased FGF21 in these pathologies likely reflects an accumulation of TG in the liver [26], but plasma FGF21 also positively correlates with the severity of steatohepatitis, particularly of fibrosis, in patients with NASH [27]. However, the induction of endogenous FGF21 levels in these states seems to be insufficient to prevent the

development of these pathologies, thus suggesting the presence of resistance to the effects of FGF21 [28], similar to insulin resistance in T2DM. Interestingly, administration of exogenous pharmacological doses of FGF21 higher than the levels found in obese mice ultimately overcomes FGF21 resistance and shows the effects of this hormone [28]. Another factor that may affect the activity of FGF21 is its proteolytic cleavage by fibroblast activation protein (FAP), an enzyme that cleaves and inactivates human FGF21 and belongs to the family of dipeptidyl peptidase-4 (DPP-4) [29]. Truncated forms of FGF21 exist in mouse plasma and differences in the activity of FAP may result in changes in the total and intact serum FGF21 levels that may influence its effects [30].

At a molecular level, FGF21 exerts its metabolic effects by binding to a receptor complex consisting of the FGF receptor (FGFR) 1c and a co-receptor called β-Klotho [31,32]. Whereas FGFR1c is ubiquitously expressed, FGF21 tissue specificity is determined by the restricted expression of β-Klotho to adipose tissue, the liver (predominantly in hepatocytes) [33], the pancreas and specific regions of the central nervous system (CNS) [34]. Activation of the FGF21 receptor complex elicits signaling cascades, including phosphorylation of the FGFR substrate-2 and the mitogen-activated protein kinase (MAPK) cascade, thus resulting in extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation [31,32].

Multiple studies have reported that FGF21 is involved in the mediation of several actions that attenuate NASH development (Figure 2). In obese rodent and monkeys, for

example, FGF21 reduces insulin levels and improves IR, one of the drivers of NAFLD/NASH [32]. These effects might be the result of the actions of FGF21 on white adipose tissue, where FGF21 reduces adiposity [35], stimulates glucose uptake by enhancing the expression of glucose transporter 1 (GLUT1) [36], modulates lipolysis [35] and increases the activity of peroxisome proliferator-activated receptor γ (PPARγ) [37]. Moreover, administration of FGF21 increases the adiponectin released by adipose tissue that mediates the beneficial effects of FGF21 on IR, hyperglycemia, dyslipidemia and steatosis in animal models of dietary or genetic obesity [38]. In addition, FGF21 also protects β-cells from apoptosis, which is likely to be associated with the glucose-lowering effect of this hormone that attenuates glucolipotoxicity [39]. Additional mechanisms by which FGF21 reduces glucose levels may also play a role, including a reduction in FFA and regulation of hepatic glucose production [35]. Thus, FGF21 acts as an acute insulin sensitizer to improve glycemic control in rodents, while also reducing insulin levels. This probably explains why FGF21 does not cause hypoglycemia even at supraphysiological levels. In line with the hepatoprotective effects of FGF21, FGF21-null mice with diabetes are more prone than wild-type mice to develop NASH [40]. However, it is worth noting that, while most of the metabolic effects of FGF21 observed in rodents and non-human primates, such as weight loss and a reduction in insulin and lipids, were reproduced in humans, no glucose-lowering effect has been observed in humans [35]. Nevertheless, studies involving humans have likely not been comprehensive enough to fully reproduce the observations reported in animal models.

In addition, FGF21 shows a potent hypotriglyceridemic effect in both animal models and humans; in the latter, plasma TG levels were reduced by approximately 50% [35].

This is remarkable, since IR and T2DM result in atherogenic dyslipidemia, which is initiated by overproduction of VLDL and is major risk factor for cardiovascular disease, the leading cause of morbidity and mortality in NASH patients.

Likewise, FGF21 mitigates lipotoxicity by promoting hepatic FA oxidation in an animal model of NASH fed with a methionine and choline-deficient (MCD) diet [41,42]. Thus, FGF21-deficient mice fed an MCD diet showed reduced hepatic FA oxidation, thus resulting in increased FFA levels. This was accompanied by more severe steatosis, peroxidative damage, inflammation and fibrosis compared to wild-type mice. Interestingly, FGF21 administration in these mice attenuated the progression to NASH. Since the MCD diet induces NASH, despite weight loss, these findings indicate that FGF21 has direct anti-inflammatory and anti-fibrotic effects that are independent of the weight loss and IR amelioration observed in obese mice. In line with the effects of FGF21 on hepatic FA oxidation, FGF21 is upregulated in the liver by peroxisome proliferator-activated receptor α (PPARα), a master regulator of hepatic FA oxidation, in response to fasting [43,44]. Moreover, the increased hepatic FA oxidation observed following the consumption of ketogenic diets is also dependent on FGF21, and its deficiency in mice fed this diet results in fatty liver with severe hypertriglyceridemia [43]. As mentioned above, FGF21 stimulates the production of adiponectin, which in turn acts in the liver to reduce hepatic ceramide levels [45], whereas the increased adiponectin seems to affect neither TG nor DAG levels.

FGF21 also attenuates hepatic ER stress, a process that contributes to hepatic steatosis, inflammation and apoptosis, and is involved in the development of NASH. Liver FGF21 expression is induced by the PKR-like ER kinase (PERK)-eukaryotic translation factor 2α (eIF2α)-activating transcription factor 4 (ATF4) branch of ER stress [46-49], where it plays a protective role by counteracting ER stress, since FGF21 deletion accelerates ER stress-induced hepatic injury and TG accumulation. The FGF21-mediated attenuation of ER stress and hepatic injury has been attributed to the activation of the AMP-activated protein kinase (AMPK)–Sirtuin1 (SIRT1) pathway by FGF21, given its protective role against ER stress, and to the attenuation of oxidative stress by FGF21 [48]. In addition, hepatic steatosis is associated with prolonged expression of C/EBP homologous protein (CHOP), a transcription factor that is upregulated by ATF4 in the context of unresolved ER stress. Interestingly, the existence of a negative feedback loop by which enhanced FGF21 expression in ER stress inhibits eIF2α, thereby reducing the expression of the ATF4-target gene CHOP, has been demonstrated [47,49]. In addition, through this negative feedback loop, FGF21 controls its own expression. Likewise, the VLDL receptor (VLDLR) is also controlled by ATF4, and the induction of ER stress provokes hepatic steatosis via the increased expression of this receptor [50]. We have previously reported that FGF21 may protect against hepatic steatosis by attenuating ER stress-induced VLDLR upregulation [51]. Furthermore, FGF21 suppresses the levels of activated SREBP1 protein maturation induced by ER stress [47].

As mentioned above, bacterial endotoxins such LPS stimulate inflammation and contribute to the development of NASH. FGF21-null mice present increased LPS-induced liver injury compared to wild-type mice, while treatment with recombinant

FGF21 can improve their survival [52], thus confirming the hepatoprotective effects of this hormone.

Regarding hepatic fibrosis, it has been reported that FGF21 administration mitigates dimethylnitrosamine (DMN)-induced hepatic fibrogenesis in mice [53]. Transforming growth factor-β (TGF-β), whose effects are mediated by the phosphorylation of Smad2 and Smad3, and the pro-inflammatory transcription factor nuclear factor (NF)-κB play a central role in the activation of hepatic stellate cells and fibrogenesis. In this study, FGF21 inhibited the activation of hepatic stellate cells by reducing the expression of TGF-β, the levels of phosphorylated Smad 2 and 3 and the activation of NF-κB by diminishing its translocation to the nucleus, whereas it increased apoptosis of these activated cells. In line with these findings, liver-targeted FGF21 gene therapy reversed HFD-induced hepatic fibrosis [54].

In addition to attenuating inflammation and fibrosis, FGF21 might also limit the progression to hepatocellular carcinoma [55]. Thus, FGF21-null mice chronically fed a high-fat, high-sucrose diet showed a deterioration of fibrosis, and 78% of the mice developed hepatocellular carcinoma, compared to only 6% of the wild-type mice. Similarly, FGF21 transfer to the liver prevented the formation of liver tumors induced by chronic HFD feeding [54].

Despite the fact that FGF21 can cross the blood-brain barrier and may be found in the cerebrospinal fluid, and that several of its effects are mediated through its actions in the CNS [56], it is currently unclear whether the effects of FGF21 on NASH involve the CNS.

Finally, several side effects have been reported after FGF21 administration [35]. For instance, a decrease in body temperature and locomotor activity has been observed in FGF21 transgenic mice but only when they were subjected to severe fasting. However, the most significant side effect reported following FGF21 administration is bone loss [35]. Thus, FGF21 transgenic mice and obese mice treated with FGF21 showed a reduction in trabecular bone volume, whereas FGF21-null mice elicited an increase in skeletal density. FGF21 induces bone loss by simultaneously decreasing bone formation and increasing bone resorption. FGF21-induced bone formation results from the inhibition of osteoblast differentiation and the switch in the differentiation of bone marrow precursors to adipocytes instead of skeletal cells [57]. The increase in bone resorption involves several indirect mechanisms, including activation of PPARγ [58], the increase in the receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) ratio, thus indicating that altered RANKL availability contributes to bone resorption [57] or induction of insulin-like growth factor binding protein 1 (IGFBP1) [59], a hepatic hormone that promotes RANKL-mediated osteoclastogenesis.

## Pharmacological strategies to modulate the effects of FGF21

The beneficial pharmacological effects of FGF21 have boosted its potential as a drug for the treatment of NASH. However, several challenges hinder the use of native FGF21 as a drug, including the need for parenteral administration and its poor pharmacokinetic properties, including a brief circulatory half-life (0.5-2 h), probably due to rapid renal clearance and proteolytic cleavage. This has led to the development of FGF21 analogs obtained by PEG(polyethylene glycol)ylation or fusion to antibody fragments. Daily

intraperitoneal administration of one of these FGF21 analogs, LY2405319, to an animal model of NASH (*ob*/*ob* mice fed an MCD diet) prevented this disease by enhancing mitochondrial function [60]. The treated mice showed improvements in metabolic abnormalities, hepatic steatosis, liver injury, and inflammatory and fibrosis markers. In addition, intraperitoneal administration of PsTag600-FGF21, a long-acting FGF21 analog, to a choline-deficient, high-fat diet-induced model of NASH reduced body weight, glucose, insulin and hepatic steatosis in a dose-dependent manner [61]. It also enhanced hepatic FA oxidation and caused a profound reduction in hepatic inflammation that was attributed to an adiponectin-dependent inhibition of interleukin (IL)-17A expression in T helper 17 (Th17) lymphocytes. In hepatocytes, DNA damage triggers inflammation via Th17 lymphocytes and IL-17A, which in turn induces adipose tissue neutrophil infiltration mediating IR and the release of FA stored in the liver as TG, thus leading to NASH and hepatocellular carcinoma [62].

LY2405319, developed by Eli Lilly, was the first FGF21 analog to be evaluated in humans [63,64]. Its daily subcutaneous administration for 28 days to patients with obesity and T2DM improved dyslipidemia and reduced body weight and plasma insulin, while increasing adiponectin levels [65]. However, only a glucose-lowering trend was observed. According to the authors of this study, treatment with LY2405319 was generally well tolerated. PF-05231023, consisting of two recombinant FGF21 molecules fused to an antibody fragment, was developed by Pfizer. This is a long-acting FGF21 analog that allows for once-weekly administration. It was intravenously administered for four weeks to obese people with hypertriglyceridemia receiving atorvastatin, with or without diabetes, and caused a marked reduction in serum TG in the absence of weight

loss [66]. Regarding safety concerns, PF-05231023 increased heart rate and blood pressure and caused modest changes in bone absorption and resorption markers, in line with an FGF21-induced bone loss effect. Although changes in markers of bone turnover might be secondary to weight loss [67], a different study with PF-05231023 reported changes in these markers in the absence of weight loss, thus indicating a direct effect of FGF21 [66]. More recently, the findings of two clinical trials with pegbelfermin (BMS-986036), a PEGylated long-acting FGF21 analog developed by Bristol-Myers Squibb that can be administered once a week, have been published. Administration of subcutaneous pegbelfermin for 12 weeks to obese and type 2 diabetic patients improved dyslipidemia, increased adiponectin and decreased the levels of the fibrosis biomarker N-terminal type III collagen propeptide (PRO-C3), without causing changes in HbA1c [68]. Confirmation of the efficacy of pegbelfermin in NASH was provided by a phase 2a clinical trial with patients suffering from this disease who received subcutaneous administration of pegbelfermin either once a day or once a week for 16 weeks [69]. Pegbelfermin treatment reduced hepatic fat fraction (more than 50% of the patients showed relative reduction of at least 30%), ameliorated dyslipidemia, increased adiponectin, and improved markers of hepatic injury and biomarkers of fibrosis (liver stiffness and PRO-C3), with no significant change in body weight. Some authors claimed that the reduction in liver fat attained by pegbelfermin is modest compared to lifestyle strategies that result in weight loss [70]. Regarding safety issues, no apparent effect on bone density, which was assessed only by bone densitometry, was observed in patients receiving pegbelfermin. However, a potential limitation of the treatment was the formation of anti-pegbelfermin and anti-FGF21 antibodies, which were observed in more than half the patients. Although the authors cited a decline in antibody titers after

the treatment, concerns have been raised about immunogenicity issues in chronic treatments with pegbelfermin [70].

Strategies in addition to FGF21 analogs are currently being developed to potentiate the effects of FGF21 that might have applications in the treatment of NASH. For example, FGF21 gene therapy has been demonstrated as an effective treatment for obesity and IR in animal models [54]. Another strategy includes the use of the non-selective FAP inhibitor talabostat [30]. Oral administration of this compound to diet-induced obese mice increased plasma FGF21 levels in obese mice, but not in lean mice. As a result of this increase, talabostat decreased body weight and improved glucose tolerance in wild-type mice, but had no effect in FGF21 knockout mice [30]. In addition, we have reported that oral compounds that activate heme-regulated (eIF2α) kinase (HRI) also increase the hepatic expression of FGF21. HRI is a kinase that phosphorylates eIF2α, which in turn increases ATF4 levels, thus resulting in enhanced FGF21 expression [49]. Oral administration of HRI activators increases FGF21 levels, ameliorates glucose intolerance and prevents liver steatosis and the increase in serum transaminases in wild-type mice fed an HFD, whereas no changes were observed in FGF21-null mice [71]. Similarly, it has been reported that promoting eIF2α phosphorylation by hepatic ablation of constitutive repressor of eIF2α phosphorylation (CReP) causes an ATF4-dependent increase in FGF21 expression that reduced body weight and ameliorated glycemic control and hepatic steatosis in mice fed a HFD [72]. Finally, NGM Bio is developing a once-monthly antibody, NGM313, which activates the β-Klotho-FGFR1c complex. A single dose of NGM313 administered to obese, insulin-resistant, non-

diabetic subjects with NAFLD caused a reduction in liver fat content, ameliorated dyslipidemia and reduced HbA1c and transaminases [73].

## Concluding Remarks and Future Perspectives

NASH is a chronic, progressive liver disease that occurs when an excessive accumulation of fat in the liver causes stress and injury to liver cells, thus leading to inflammation and fibrosis, which can progress to cirrhosis, liver failure, cancer and eventually death. There is compelling evidence that FGF21 may be an attractive target for the treatment of NASH. FGF21 and its analogs stimulate hepatic FA oxidation, thereby decreasing hepatic fat accumulation, and improving IR, one of the main drivers of NASH. In addition, FGF21 raises levels of adiponectin, an adipokine with insulin-sensitizing, anti-steatotic, anti-inflammatory and anti-fibrotic effects [17]. A recent phase 2a clinical trial has demonstrated that administration of the FGF21 analog pegbelfermin to patients with NASH reduces hepatic fat content. However, it is unclear how this reduction in hepatic fat content affects liver-related outcomes and, since there are no universally accepted surrogate endpoints for NASH, additional studies with a larger number of patients should confirm the effects of this analog on liver histology and clinical outcomes. In addition, safety concerns, including cardiovascular side effects and bone loss (see Outstanding Questions), have been raised for some FGF21 analogs, and questions remain regarding the safety of chronic treatment with FGF21 analogs.

## Acknowledgments

We apologize to any contributors to this field of research whose work is not cited here due to space restrictions. This work was funded by the Spanish *Ministerio de Economía*

*y Competitividad* of the Spanish Government (SAF2015-64146-R and RTI2018-093999-B-100) and *CIBER de Diabetes y Enfermedades Metabólicas Asociadas* (CIBERDEM). CIBERDEM is an initiative of the Instituto de Salud Carlos III (ISCIII) - Ministerio de Economía y Competitividad. We thank the University of Barcelona's Language Advisory Service for revising the manuscript.

## Disclosure statement

M.Z. and M.V-C. are co-inventors of the patent titled "HRI activators useful for the treatment of cardiometabolic diseases" derived from WO2018/010856.

## References

1. Arab, J.P., Arrese, M. and Trauner, M. (2018) Recent Insights into the Pathogenesis of Nonalcoholic Fatty Liver Disease. *Annu. Rev. Pathol.* 13, 321-350.
2. Brunt, E.M. *et al.* (2015) Nonalcoholic fatty liver disease. *Nat. Rev. Dis. Primers* 1, 15080.
3. European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO) (2016) EASL-EASD-EASO Clinical practice guidelines for the management of non-alcoholic fatty liver disease. *J. Hepatol.* 64, 1388–402
4. Lindenmeyer, C.C. and McCullough, A.J. (2018) The Natural History of Nonalcoholic Fatty Liver Disease-An Evolving View. *Clin. Liver Dis.* 22, 11-21.
5. Kim D, et al. (2019) Changing trends in etiology- and ethnicity-based annual mortality rates of cirrhosis and hepatocellular carcinoma in the United States. *Hepatology* 69, 1064-1074.
6. Younossi, Z.M. (2019) Non-alcoholic fatty liver disease - A global public health perspective. *J. Hepatol.* 70, 531–544.
7. Alonso, C., *et al.* (2017) Metabolomic Identification of Subtypes of Nonalcoholic Steatohepatitis. *Gastroenterology* 152:1449-1461.

8. Bril, F. *et al.* (2017) Metabolic and histological implications of intrahepatic triglyceride content in nonalcoholic fatty liver disease. *Hepatology* 65:1132-1144.
9. Kawano, Y. *et al.* (2015) Identification of Lipid Species Linked to the Progression of Non-Alcoholic Fatty Liver Disease. *Curr. Drug Targets* 16: 1293-300.
10. Luukkonen, P.K. *et al.* (2016) Hepatic ceramides dissociate steatosis and insulin resistance in patients with non-alcoholic fatty liver disease. *J. Hepatol.* 64:1167-1175.
11. Tsuchida, T. and Friedman, S.L. (2017) Mechanisms of hepatic stellate cell activation. *Nat. Rev. Gastroenterol. Hepatol.* 14: 397-411.
12. Romeo, S., *et al.* (2008) Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat. Genet.* 40:1461-1465.
13. Bruschi, F.V. *et al.* (2017) The PNPLA3 I148M variant modulates the fibrogenic phenotype of human hepatic stellate cells. Hepatology 65:1875-1890.
14. Marra, F. and Svegliati-Baroni, G. (2017) Lipotoxicity and the gut-liver axis in NASH pathogenesis. *J. Hepatol.* 68:280-295.
15. Gan, L.T. *et al.* (2014) Hepatocyte free cholesterol lipotoxicity results from JNK1-mediated mitochondrial injury and is HMGB1 and TLR4-dependent. *J. Hepatol.* 61:1376-84.
16. Polyzos, S.A. *et al.* (2016) Adipokines in nonalcoholic fatty liver disease. *Metabolism* 65: 1062-79.
17. Saxena, N.K. and Anania, F.A. (2015) Adipocytokines and hepatic fibrosis. *Trends Endocrinol. Metab.* 26:153-61.
18. Nishimura, T. *et al.* (2000) Identification of a novel FGF, FGF-21, preferentially expressed in the liver. *Biochim. Biophys. Acta* 1492:203-206.
19. Markan, K.R. *et al.* (2014) Circulating FGF21 is liver derived and enhances glucose uptake during refeeding and overfeeding. *Diabetes* 63:4057-4063.
20. Hondares, E. *et al.* (2014) Fibroblast growth factor-21 is expressed in neonatal and pheochromocytoma-induced adult human brown adipose tissue. *Metabolism* 63:312-317.
21. Singhal, G. *et al.* (2016) Fibroblast Growth Factor 21 (FGF21) Protects against High Fat Diet Induced Inflammation and Islet Hyperplasia in Pancreas. *PLoS One* 11: e0148252.
22. Suomalainen, A. (2013) Fibroblast growth factor 21: a novel biomarker for human muscle-manifesting mitochondrial disorders. *Expert Opin. Med. Diagn.* 7:313-317.
23. Zhang, X. *et al.* (2008) Serum FGF21 levels are increased in obesity and are independently associated with the metabolic syndrome in humans. *Diabetes* 57:1246-1253.
24. Mraz, M. *et al.* (2009) Serum concentrations and tissue expression of a novel endocrine regulator fibroblast growth factor-21 in patients with type 2 diabetes and obesity. *Clin. Endocrinol.* 71: 369-375.

25. Dushay, J. *et al.* (2010) Increased fibroblast growth factor 21 in obesity and nonalcoholic fatty liver disease. *Gastroenterology* 139:456-463.
26. Li, H. *et al.* (2010) Fibroblast growth factor 21 levels are increased in nonalcoholic fatty liver disease patients and are correlated with hepatic triglyceride. *J. Hepatol.* 53:934-940.
27. Barb, D. et al. (2019) Plasma Fibroblast Growth Factor 21 Is Associated With Severity of Nonalcoholic Steatohepatitis in Patients With Obesity and Type 2 Diabetes. *J. Clin. Endocrinol. Metab.* 104:3327-3336.
28. Fisher, F.M. *et al.* (2010) Obesity is a fibroblast growth factor 21 (FGF21)-resistant state. *Diabetes* 59:2781-2789.
29. Dunshee, D.R. *et al.* (2016) Fibroblast Activation Protein Cleaves and Inactivates Fibroblast Growth Factor 21. *J. Biol. Chem.* 291:5986-5996.
30. Sánchez-Garrido, M.A. *et al.* (2016) Fibroblast activation protein (FAP) as a novel metabolic target. *Mol. Metab.* 5:1015-1024.
31. Kurosu, H. *et al.* (2007) Tissue-specific expression of betaKlotho and fibroblast growth factor (FGF) receptor isoforms determines metabolic activity of FGF19 and FGF21. *J. Biol. Chem.* 282:26687-26695.
32. Ogawa, Y. *et al.* (2007) BetaKlotho is required for metabolic activity of fibroblast growth factor 21. *Proc. Natl. Acad. Sci. U S A* 104:7432-7437.
33. Kobayashi, K. *et al.* (2016) Hepatocyte β-Klotho regulates lipid homeostasis but not body weight in mice. *FASEB J.* 30:849-862.
34. Fon Tacer, K. *et al.* (2010) Research resource: Comprehensive expression atlas of the fibroblast growth factor system in adult mouse. *Mol. Endocrinol.* 24:2050-2064.
35. Kharitonenkov, A. and DiMarchi, R. (2017) Fibroblast growth factor 21 night watch: advances and uncertainties in the field. *J. Intern. Med.* 281:233-246.
36. Ge, X. *et al.* (2011) Fibroblast growth factor 21 induces glucose transporter-1 expression through activation of the serum response factor/Ets-like protein-1 in adipocytes. *J. Biol. Chem.* 286:34533-34541.
37. Murata, Y. *et al.* (2011) FGF21 as an Endocrine Regulator in Lipid Metabolism: From Molecular Evolution to Physiology and Pathophysiology. *J. Nutr. Metab.* 2011:981315.
38. Hui, X. (2016) The FGF21-adiponectin axis in controlling energy and vascular homeostasis. *J. Mol. Cell Biol.* 8:110-119.
39. Lin, X. *et al.* (2017) Metabolic role of fibroblast growth factor 21 in liver, adipose and nervous system tissues. *Biomed. Rep.* 6:495-502.
40. Liu, X. *et al.* (2016) Lack of fibroblast growth factor 21 accelerates metabolic liver injury characterized by steatohepatities in mice. *Am. J. Cancer Res.* 6:1011-25.
41. Fisher, F.M. *et al.* (2014) Fibroblast growth factor 21 limits lipotoxicity by promoting hepatic fatty acid activation in mice on methionine and choline-deficient diets. *Gastroenterology* 147:1073-1083.

42. Tanaka, N. *et al.* (2015) Role of fibroblast growth factor 21 in the early stage of NASH induced by methionine- and choline-deficient diet. *Biochim. Biophys. Acta* 1852:1242-52.
43. Badman, M.K. *et al.* (2007) Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. *Cell Metab.* 2007 5:426-437.
44. Inagaki, T. *et al.* (2007) Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. *Cell Metab.* 5:415-425.
45. Holland, W.L. et al. (2013) An FGF21-adiponectin-ceramide axis controls energy expenditure and insulin action in mice. *Cell Metab.* 17:790-797.
46. De Sousa-Coelho, A.L., Marrero, P.F., Haro, D. (2012) Activating transcription factor 4-dependent induction of FGF21 during amino acid deprivation. *Biochem. J.* 443:165-171.
47. Jiang, S. *et al.* (2014) Fibroblast growth factor 21 is regulated by the IRE1α-XBP1 branch of the unfolded protein response and counteracts endoplasmic reticulum stress-induced hepatic steatosis. *J. Biol. Chem.* 289:29751-29765.
48. Kim, S.H. et al. (2015) Fibroblast growth factor 21 participates in adaptation to endoplasmic reticulum stress and attenuates obesity-induced hepatic metabolic stress. *Diabetologia* 58:809-818.
49. Zarei, M. *et al.* (2016) Heme-Regulated eIF2α Kinase Modulates Hepatic FGF21 and Is Activated by PPARβ/δ Deficiency. *Diabetes* 65:3185-3199.
50. Jo, H. *et al.* (2013) Endoplasmic reticulum stress induces hepatic steatosis via increased expression of the hepatic very low-density lipoprotein receptor. *Hepatology* 57:1366-1377.
51. Zarei, M. *et al.* (2018) Hepatic regulation of VLDL receptor by PPARβ/δ and FGF21 modulates non-alcoholic fatty liver disease. *Mol. Metab.* 117-131.
52. Kenneth, R. *et al.* (2012) FGF21 Is Increased by Inflammatory Stimuli and Protects Leptin-Deficient ob/ob Mice from the Toxicity of Sepsis. *Endocrinology* 153: 2689–2700.
53. Xu. P. *et al.* (2016) Fibroblast growth factor 21 attenuates hepatic fibrogenesis through TGF-β/smad2/3 and NF-κB signaling pathways. *Toxicol. Appl. Pharmacol.* 290:43-53.
54. Jimenez, V. *et al.* (2018) FGF21 gene therapy as treatment for obesity and insulin resistance. *EMBO Mol. Med.* 10: e8791.
55. Singhal, G. *et al.* (2018) Deficiency of fibroblast growth factor 21 (FGF21) promotes hepatocellular carcinoma (HCC) in mice on a long term obesogenic diet. *Mol. Metab.* 13:56-66.
56. Bookout, A.L. *et al.* (2013) FGF21 regulates metabolism and circadian behavior by acting on the nervous system. *Nat. Med.* 19:1147-1152.
57. Wei, W. *et al.* (2012) Fibroblast growth factor 21 promotes bone loss by potentiating the effects of peroxisome proliferator-activated receptor γ. *Proc. Natl. Acad. Sci. U S A* 109: 3143–3148.

558 58. Paul, A. et al. (2012) Fibroblast Growth Factor-21 Regulates PPARγ Activity
559 and the Antidiabetic Actions of Thiazolidinediones Cell 148:556–567.
560 59. Wang, X. et al. (2015) A Liver-Bone Endocrine Relay by IGFBP1 Promotes
561 Osteoclastogenesis and Mediates FGF21-Induced Bone Resorption. *Cell Metab.*
562 22: 811–824.
563 60. Lee, J.H. *et al.* (2016) An engineered FGF21 variant, LY2405319, can prevent
564 non-alcoholic steatohepatitis by enhancing hepatic mitochondrial function. *Am.*
565 *J. Transl. Res.* 8:4750-4763.
566 61. Bao, L. *et al.* (2018) A long-acting FGF21 alleviates hepatic steatosis and
567 inflammation in a mouse model of non-alcoholic steatohepatitis partly through
568 an FGF21-adiponectin-IL17A pathway. *Br. J. Pharmacol.* 175:3379-3393.
569 62. Gomes, A.L. *et al.* (2016) Metabolic Inflammation-Associated IL-17A Causes
570 Non-alcoholic Steatohepatitis and Hepatocellular Carcinoma. *Cancer Cell*
571 30:161-175.
572 63. Kharitonenkov, A., and Adams, A.C. (2013) Inventing new medicines: The
573 FGF21 story. *Mol. Metab.* 3:221-229.
574 64. Struik, D. *et al.* (2019) Fibroblast growth factors in control of lipid metabolism:
575 from biological function to clinical application. *Curr. Opin. Lipidol.* 30:235-243.
576 65. Gaich, G. *et al.* (2013) The effects of LY2405319, an FGF21 analog, in obese
577 human subjects with type 2 diabetes. *Cell Metab.* 18:333-340.
578 66. Kim, A.M. *et al.* (2017) Once-weekly administration of a long-acting fibroblast
579 growth factor 21 analogue modulates lipids, bone turnover markers, blood
580 pressure and body weight differently in obese people with hypertriglyceridaemia
581 and in non-human primates. *Diabetes Obes. Metab.*19:1762-1772.
582 67. Talukdar, S. *et al.* (2016) A Long-Acting FGF21 Molecule, PF-05231023,
583 Decreases Body Weight and Improves Lipid Profile in Non-human Primates and
584 Type 2 Diabetic Subjects. *Cell Metab.* 23:427-440.
585 68. Charles, E.D. *et al.* (2019) Pegbelfermin (BMS-986036), PEGylated FGF21, in
586 Patients with Obesity and Type 2 Diabetes: Results from a Randomized Phase 2
587 Study. Obesity 27:41-49.
588 69. Sanyal, A. *et al.* (2019) Pegbelfermin (BMS-986036), a PEGylated fibroblast
589 growth factor 21 analogue, in patients with non-alcoholic steatohepatitis: a
590 randomised, double-blind, placebo-controlled, phase 2a trial. *Lancet* 392: 2705-
591 2717.
592 70. Dushay, J., Lai, M. (2019) Is Trimming the Fat Enough? Fibroblast Growth
593 Factor 21 as An Emerging Treatment for Nonalcoholic Fatty Liver Disease.
594 *Hepatology* 70:1860-1862.
595 71. Zarei, M. *et al.* (2019) Oral administration of a new HRI activator as a new
596 strategy to improve high-fat-diet-induced glucose intolerance, hepatic steatosis,
597 and hypertriglyceridaemia through FGF21. *Br. J. Pharmacol.* 176:2292-2305.
598 72. Xu, X. *et al.* (2018) Preemptive Activation of the Integrated Stress Response
599 Protects Mice From Diet-Induced Obesity and Insulin Resistance by Fibroblast
600 Growth Factor 21 Induction. *Hepatology* 68:2167-2181.

73. Shankar, S. *et al.* (2018) NGM313, a novel once-monthly activator of b klotho-FGFR1c, significantly reduces hepatic steatosis and key biomarkers of nonalcoholic steatohepatitis. Poster presented at: The Liver Meeting of The American Association for the Study of Liver Diseases (AASLD); San Francisco, 2018.

## FIGURE LEGENDS

**Figure 1. Schematic summary of the pathophysiology of NASH.**

The main drivers of the development of NASH are obesity and insulin resistance, favored by the consumption of fat- or fructose-rich diets. Genetic predisposition also plays a key role. As visceral obesity develops in the context of insulin resistance, it results in greater release of FFA, since insulin resistance leads to the failure of lipolysis inhibition by this hormone. Enhanced delivery of FFA to the liver results in lipotoxicity, mitochondrial dysfunction, ROS generation, lipid peroxidation, ER stress and inflammation, which ultimately result in HSC activation and fibrogenesis. NASH can progress to cirrhosis, liver failure, cancer and eventually death.

DAG: diacylglycerol; DNL: *de novo* lipogenesis; ER: endoplasmic reticulum; FFA: free fatty acids; HSC: hepatic stellate cell; LPC: lysophosphatidylcholine; LPS: lipopolysaccharide; ROS: reactive oxygen species; TG: triglycerides; VLDL: very low-density lipoproteins.

**Figure 2. Pharmacological approaches to target FGF21 and its main effects in NASH.** Potential pharmacological approaches to treat NASH (FGF21 analogs, oral inducers of native FGF21 such as HRI activators and FAP inhibitors, and antibodies that activate the β-Klotho-FGFR1c complex) based on FGF21 and their potential beneficial effects on this disease are depicted.

ATF4: activating transcription factor 4; eIF2α: eukaryotic translation factor 2α; FAP: fibroblast activation protein; FGFR1c: fibroblast growth factor receptor 1c; HRI: heme-regulated (eIF2α) kinase; KLB: β-Klotho; TG: triglycerides.

**Glossary**

**Adipokines (Adipocytokines):** peptides produced by adipose tissue that exert autocrine, paracrine and endocrine function.

**Atherogenic dyslipidemia:** the presence of high levels of triglycerides, small-dense low-density lipoprotein, and low levels of high-density lipoprotein cholesterol. It is often observed in patients with metabolic syndrome, obesity, insulin resistance and T2DM, and NAFLD.

**Cytokine:** a small protein secreted by cells that has a specific effect on the interactions and communication between cells.

**ER stress:** the result of any stimulus that provokes the accumulation of misfolded proteins in the lumen of the ER. ER stress triggers the unfolded protein response (UPR), an adaptive (defensive) ER-stress response that involves activation of a signaling pathway to restore folding capacity. If ER homeostasis is not restored, inflammation and apoptosis is induced**.**

**Fibrosis:** the excessive accumulation of extracellular matrix that often occurs in response to chronic tissue injury, and may cause disruption of organ architecture and loss of function.

**FFA:** FA released from triglycerides by the action of the enzyme lipase and transported in the blood bound to albumin.

**Hepatocyte ballooning:** special form of liver cell degeneration that is defined by hematoxylin and eosin staining showing enlarged, swollen hepatocytes with loss of the usual polygonal shape of the cell.

**Hepatic stellate cells**: liver-specific mesenchymal cells that play vital roles in liver physiology and fibrogenesis. Once activated, stellate cells produce extracellular matrix that generate a temporary scar at the site of injury to protect the liver from further damage. Prolonged and repeated activation of stellate cells causes liver fibrosis that may cause scar formation and disruption of liver architecture and function.

**Hepatokines**: proteins secreted by hepatocytes that can influence metabolic processes.

**Kupffer cells:** critical component of the immune system implicated in both liver injury and repair. They exhibit tremendous plasticity, expressing a range of polarized macrophages, from the proinflammatory M1 to the alternative/M2 phenotype, which is involved in the resolution of inflammation and wound healing.

**Lipotoxicity:** when excess lipids in non-adipose tissues are driven into alternative non-oxidative pathway that promotes metabolically relevant cellular dysfunction.

**Mitochondrial dysfunction:** loss of efficiency in the electron transport chain and reductions in the synthesis of high-energy molecules, such as adenosine-5′-triphosphate (ATP). It is characteristic of aging, and essentially, of all chronic diseases.

## Outstanding Questions

Will FGF21 analogs improve liver histology in patients with NASH?

Will FGF21 analogs show a reduction in clinical outcomes in patients with NASH?

How would potential side effects affect the development of FGF21 analogs for the treatment of NASH?

Will FGF21 analogs be safe in chronic treatments for NASH?

Are pharmacological approaches in study an option to obtain oral treatments to target FGF21 in NASH?

**Comments for Reviewer 1**

We are very grateful to the comments of the reviewer (*Zarei et al. nicely summarized the effects of FGF21 on NASH. The paper reads very well).* In addition, we would like to thank the reviewer for his/her useful suggestions, which have allowed us to include changes to improve the manuscript.

**Comments:**

***Comment 1****. On page 8, line 182, the authors stated the restricted expression of beta-Klotho to the liver. The authors described KLB on hepatocytes in the figure. To my knowledge, the data regarding the expression of KLB are controversial. Please clarify this in the paper.*

Although KLB is highly expressed in liver of mice (reference 33 of the manuscript) and humans (please, see Gut and Liver 12: 449-456, 2018), the cell type that express KLB in liver was not identified until recently. Kobayashi et al. (please, see FASEB J. 30: 849–862, 2016) recently examined the expression of KLB in hepatocyte and non-hepatocyte fractions freshly isolated from liver. They found that KLB was predominantly expressed in hepatocytes.

According to the suggestion of the reviewer, we have included a comment to indicate that KLB is predominantly expressed in hepatocytes (please, see page 8, line 183).

***Comment 2****. Several recent articles regarding FGF21 and NASH should be cited.*

*Dushay J, Lai M.: Is Trimming the Fat Enough? Fibroblast Growth Factor 21 as An Emerging Treatment for Nonalcoholic Fatty Liver Disease. Hepatology. 2019;70(5):1860-1862*

*Xu X, Krumm C, So JS, Bare CJ, Holman C, Gromada J, Cohen DE, Lee AH. Preemptive Activation of the Integrated Stress Response Protects Mice From Diet-Induced Obesity and Insulin Resistance by Fibroblast Growth Factor 21 Induction. Hepatology. 2018;68(6):2167-2181.*

According to the suggestion of the reviewer, we have included two comments in the revised version of the manuscript that include these references. Please, see page 15 (lines 348-350 and 355-356) and page 16 (lines 372-375).

**Comments for Reviewer 2**

We are very grateful to the comments of this reviewer (*This manuscript is well written and informative for readers.*).