UNIVERSITAT DE BARCELONA

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"The role of hepatic FGF21 (*Fibroblast Growth Factor 21)* in the maintenance of metabolic homeostasis during metabolic stress"

Thesis submitted by Úrsula Monserrat Martínez Garza to opt for the title of doctor for the University of Barcelona

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This thesis was supported by a grant awarded by the National Council of Science and Technology (CONACYT).

A mis padres, Helio y Marcela

por siempre estar tan cerca de mi
aún estando del otro lado del mundo

porque me enseñaron a nunca rendirme
y a dar lo mejor de mi

por siempre estar disponibles para mi
aún con 7h de diferencia horaria

por sacarme mil sonrisas
y hacerme sentir tan querida

por apoyarme en cada decisión
y en cada etapa de mi vida

por ser mis mejores amigos
y las personas que más admiro en el mundo

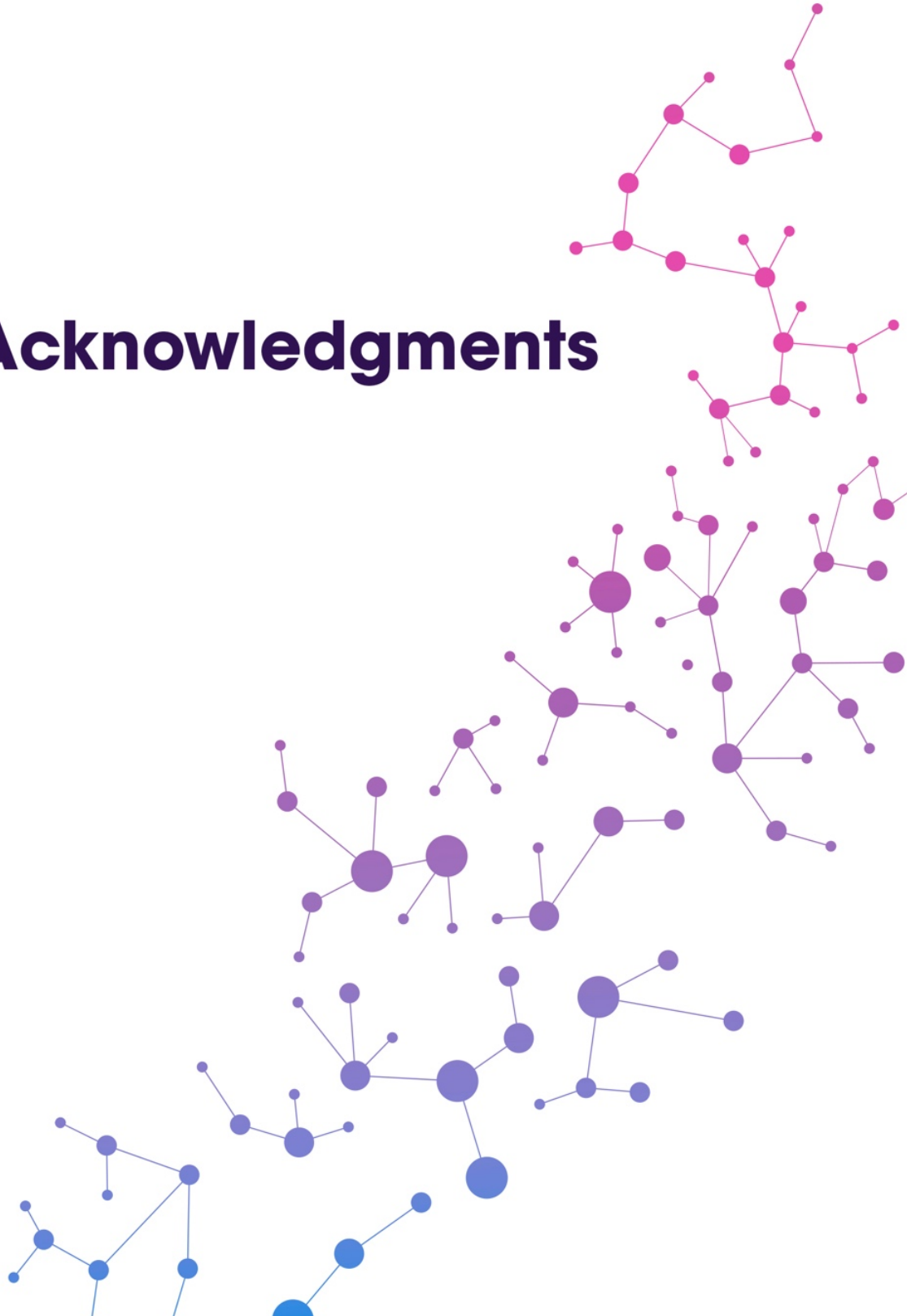
sin ustedes ésto no hubiera sido posible

con todo mi cariño,

les dedico mi tesis doctoral

Úrsula

Acknowledgments



A mis hermanos Helio y Aarón,

Ustedes dos me han enseñado más de la vida que cualquier doctorado que pueda hacer.

Aarón gracias por compartirme tu música y tus creaciones, escucharte cantar por las mañanas siempre alegró mis mañanas de camino al lab.

Helio gracias por todos los mensajes que me enviaste cada día por los últimos 5 años. Sentí en cada momento tu apoyo y tu cariño.

Gracias por haber estado esperándome en el aeropuerto cada vez que llegaba a Monterrey (aún y cuando el vuelo se retrasara muchas horas). Verlos allí esperándome con un gran abrazo de oso es lo que más esperaba al volver a casa.

Gracias por estar siempre para mi y ser mi alegría.

Úrsula

Esta tesis además de ser un largo trabajo de investigación, también abarca un gran camino de conocimientos aprendidos, de horas de trabajo, de fallas, victorias, tristezas y alegrías. Mil historias y experiencias que llenan cada una de las páginas que contiene este libro.

Esta tesis representa una etapa de mi vida donde aprendí a valerme por mi misma en un país extranjero. Nadie dijo que fuera fácil y definitivamente no lo fue, pero también fue la mejor experiencia transformadora que he tenido en la vida.

He aprendido sobre una nueva cultura, a ser fuerte pero también a ser vulnerable. Aprendí a pedir ayuda, aunque me costó mucho algunas veces. Aprendí a quererme y a cuidarme, pero también a querer y cuidar a las personas valiosas que llegan a mi vida. Aprendí que los amigos a veces se vuelven familia y sin duda yo he tenido a la mejor familia en Barcelona.

Llegué a Barcelona hace 5 años a empezar mis estudios de posgrado, llegué sola pero nunca me sentí sola, personas increíbles me han acompañado en este viaje del doctorado y quiero mencionar a cada uno lo valiosa que ha sido su compañía durante este tiempo.

Primero, a Sara y Albert (también Leo y Ari), mi familia en Barcelona. Agradezco tanto a Idealista y a la vida el que nos hayamos conocido, por mucho ustedes han sido de las personas más maravillosas que he conocido. Gracias por cuidarme tantas

veces, por acompañarme y hacerme sentir en familia. Por ese arroz al horno de Albert de los findes y a Sara por tantas cervecitas en las terrazas e interminables conversaciones, por ser la mejor compañía de Barcelona. Son lo máximo los dos.
A Brenda, mi querida amiga, tu has sido mi hermana desde que pisé Barcelona hace 5 años. Gracias por tus consejos y porque siempre me has hecho sentir como en mi casa en México. Eres increíble y te agradezco por tantos momentos juntas y por tu amistad.

A mis compañeros del laboratorio, Hector y Vivi gracias por enseñarme tanto, por su apoyo y por hacer que trabajar en el lab fuera mucho más feliz y divertido. Vivi gracias por ser como mi hermana, por tus consejos y por compartir tanta de tu alegría conmigo.

A Dani Torres, gracias por comprarme de mis galletitas favoritas para desayunar, por la buena conversación y el cafecito de las mañanas, eres un gran amigo y has hecho que mi tiempo en el laboratorio sea muy feliz. Gracias por tu amistad.

A Alex, siempre fuiste un gran apoyo. Alex tu eres súper brillante y cuando a mi no se me prendía el foco a ti si y siempre hicimos un gran equipo. Gracias por acompañarme tantas veces y llevarme a mi casa cuando me sentí mal. También eres el mejor Dj de música latina en Barcelona, lo tengo que aceptar. Eres lo máximo. Gracias por todo Alex.

A todos mis amigos del doctorado, Bea, Sonia, Oriol, Salva, Nelly, Josè, Sara, Fer y Mariana, gracias por tan buena conversación cada hora de comer, por los chocolates, el café y los viajes. Ha sido increíble compartir con ustedes estos años y éste proceso, siempre los voy a admirar y los recordaré con mucho cariño.

Quiero agradecer a mi director Diego y a mi tutor Pedro, por guiarme y compartirme todo lo que saben. Me ha encantado tenerlos a los dos como guías y escuchar sus discusiones cada seminario, su sentido del humor siempre alegró las juntas de equipo. Gracias por su apoyo todos estos años.

Finalmente, quiero agradecer a Joana, mi directora de tesis. Joana en estos años me has enseñado muchísimo. Gracias por ser tan paciente, se que muchas veces estuve perdida y que me costó adaptarme al lab al principio pero tu siempre tuviste ganas de enseñarme y de guiarme cada vez. Gracias por estar siempre disponible para mi cada que entraba a tu despacho (un millón de veces) y por preocuparte por mi. Joana eres una persona admirable, una gran profesora y una brillante investigadora. Gracias por todo tu apoyo y tus enseñanzas. No pude haber tenido mejor directora. Has hecho que estos años de formación hayan sido una gran experiencia. Te admiro y te aprecio mucho.

Todo lo que empieza llega a su final y hoy yo acabo esta etapa con una gran sonrisa por todo lo aprendido y por todas las grandes experiencias que se quedan guardadas entre líneas en este libro.

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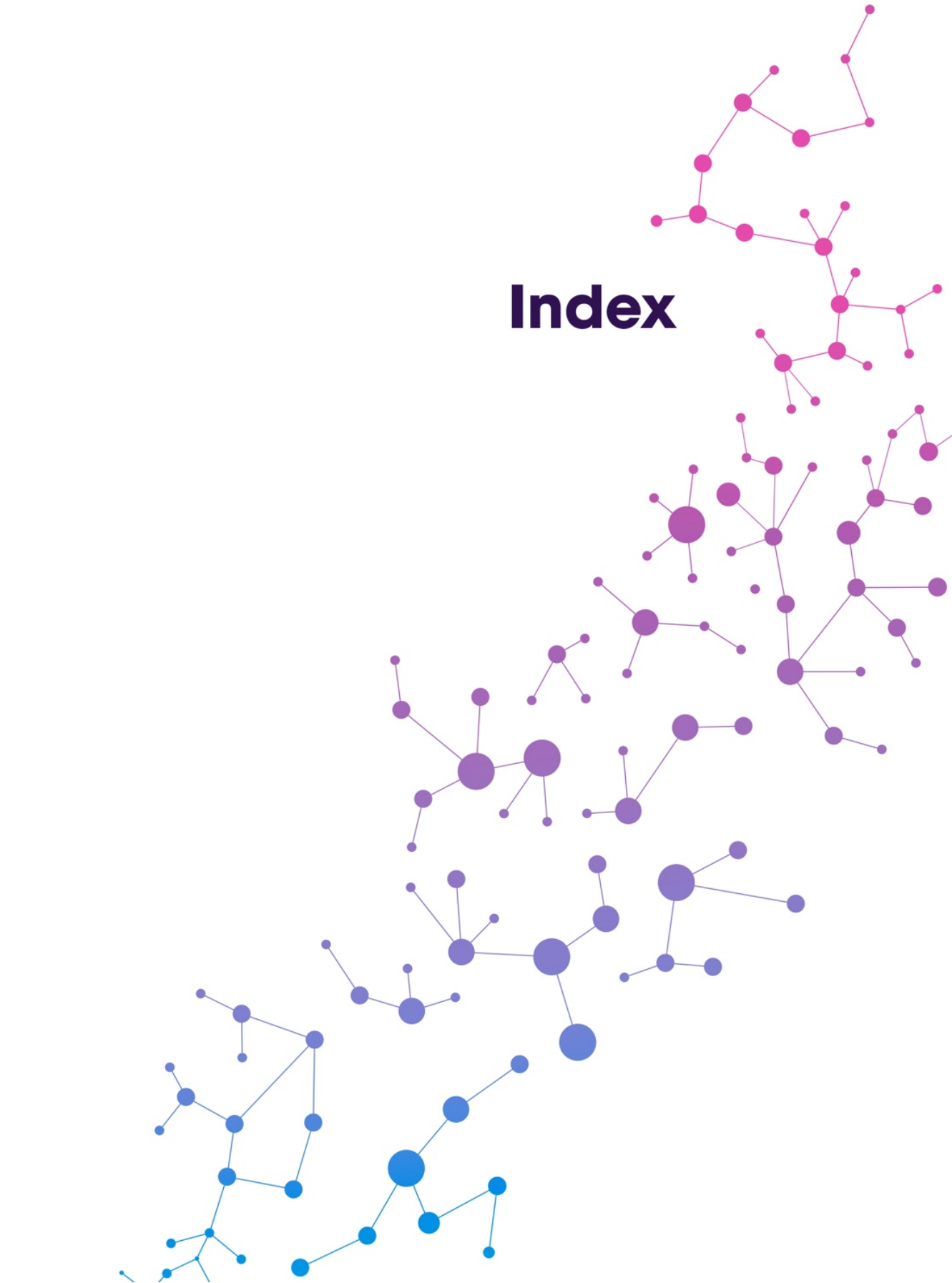
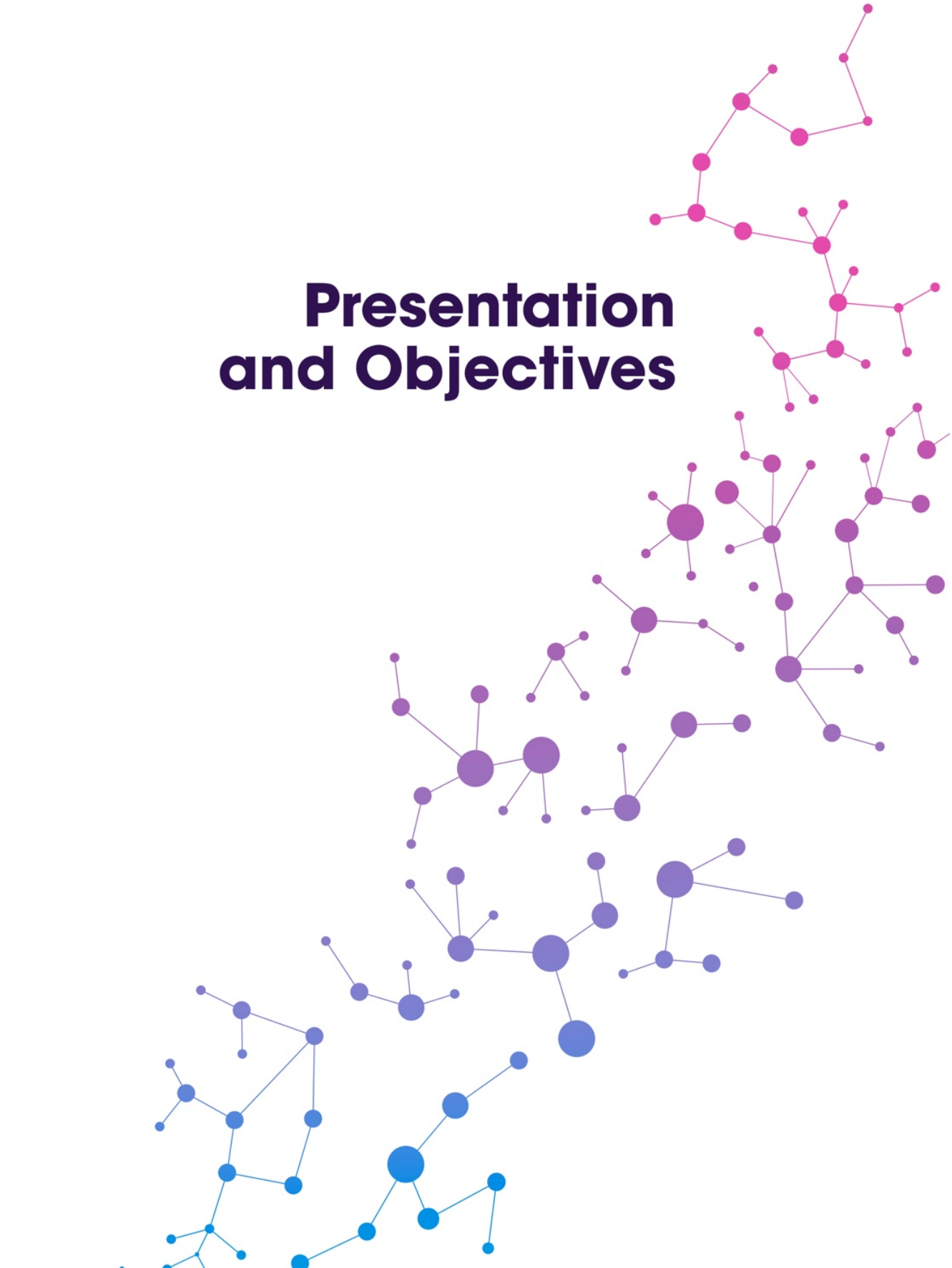


Presentation and Objectives



The excessive consumption of calorie-rich foods, together with a sedentary lifestyle, drive the actual global obesity pandemic. Obesity is the leading cause of several other metabolic disorders, such as diabetes, cardiovascular diseases, cancer, and non-alcoholic fatty liver disease (NAFLD). NAFLD is one of the most important causes of liver disease worldwide; the global prevalence of NAFLD is currently estimated to be 24% [1] . It begins with simple steatosis and can progress to various states of fibrosis, causing severe damage and dysfunction [2]. As the incidence rates of these metabolic pathologies continue increasing worldwide, novel molecular and nutritional approaches had emerged as therapeutic candidates to treat obesity-related pathologies as NAFLD.

Fibroblast growth factor 21 (FGF21) has been described as an anti-obesity and antidiabetic hormone because of its potent effects over glucose and lipid metabolism [3]. Also, recent publications suggest that FGF21 effects on the liver lead to a reduction in liver fat content and decrease fibrosis and inflammation [4,5]. For these reasons, FGF21 is considered as a candidate to treat obesity-related metabolic disorders.

FGF21 is a protein secreted mainly by the liver but also by other tissues or organs such as white adipose tissue (WAT), brown adipose tissue (BAT), intestine, skeletal and cardiac muscle and pancreas [6]. FGF21 constitutes a central component of the endocrine/autocrine system; it exerts pleiotropic effects and acts as a potent metabolic regulator being a critical factor in maintaining the energy homeostasis [7–9].

FGF21 has been identified as a sensitive nutritional hormone. Some of our previous results show that mice fed with a low protein diet (5% of protein intake) for seven days increased Fgf21 expression in the liver. Protein restriction mediated by hepatic FGF21 promoted weight loss and induced

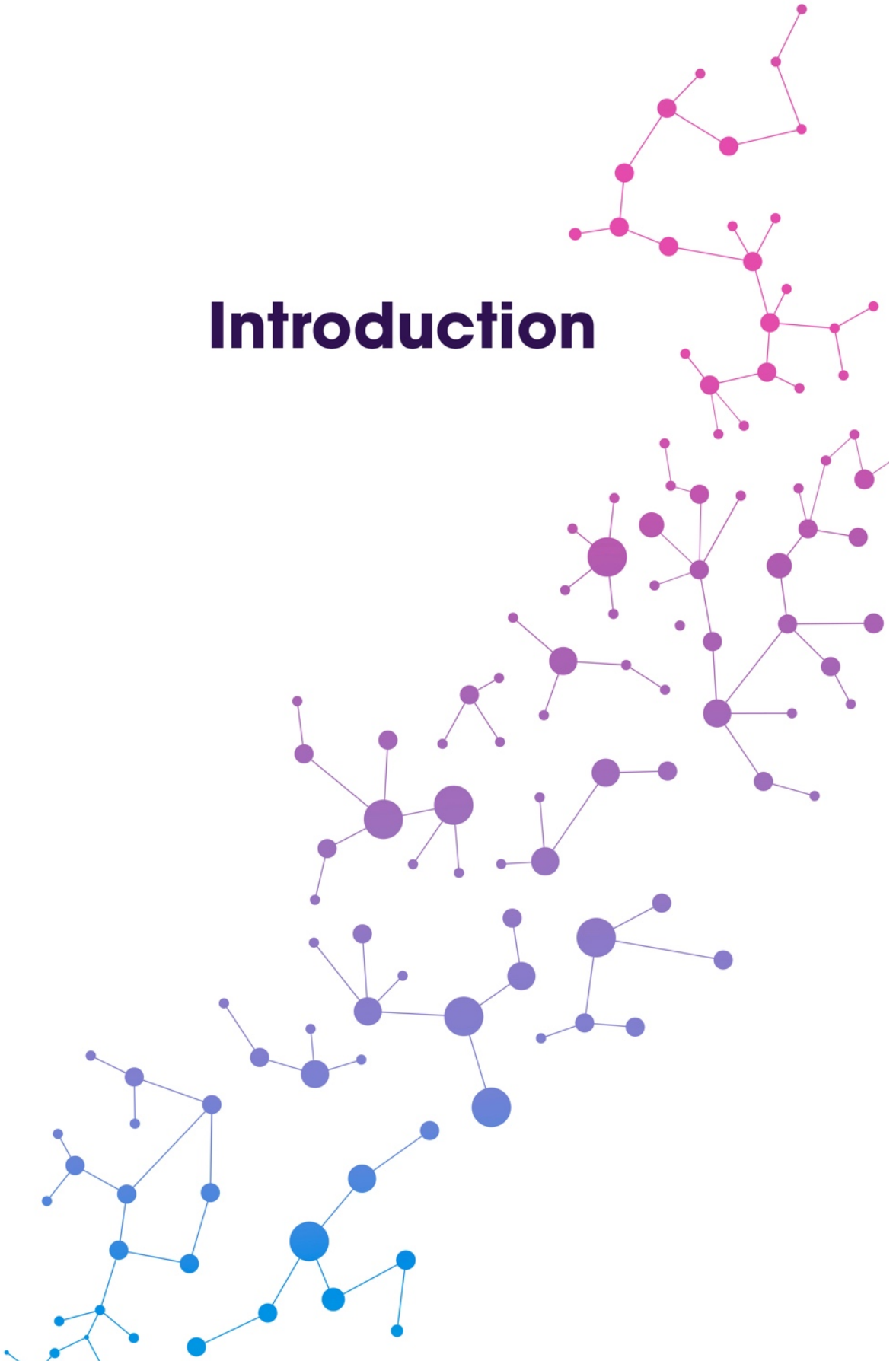
browning in subcutaneous white adipose tissue (scWAT) by an increase of uncoupling protein 1 (UCP1) expression [10]. Other authors demonstrated that reduced protein intake restored glucose tolerance, preventing the onset of hyperglycemia and beta-cell loss and restored insulin sensibility in mice [11,12].

Recent findings suggest that the metabolic responses, induced by protein restriction, as effects over macronutrient preference, growth, and glucose homeostasis, are mediated by FGF21 signaling in the brain [13]. A low protein diet increases the GCN2-dependent phosphorylation of eIF2a, resulting in higher ATF4 protein levels [14]. ATF4 is a transcription factor that induces the FGF21 expression by binding two conserved functional ATF4-binding sequences situated in the Fgf21 promoter [14,15].

Because of the effects of FGF21 over lipid metabolism, the hypothesis in this work is that FGF21 may protect the liver during an acute or chronic fat deposition in this tissue. Also, because of the positive effects of protein restriction, we considered that a low protein diet, mediated by hepatic FGF21, may be protective and ameliorate the adverse metabolic effects produced by high-fat diet-induced obesity.

The global aim of this project is to understand the role of hepatic FGF21 in the maintenance of metabolic homeostasis during high metabolic stress. This project's specific objectives are: 1) To study the effects of hepatic FGF21 on lipid homeostasis due to excessive acute fat storage caused by tunicamycin. 2) To analyze the effects of protein restriction on protecting against and counteracting the effects of a high lipid intake. 3) To understand the role of FGF21 in the metabolic response to fasting.

Introduction



Fibroblast Growth Factor 21

The Fibroblast Growth Factor (FGF) family comprises 23 structurally related proteins divided into seven sub-families due to their phylogenetic similarity. They show pleiotropic effects on development, organogenesis, cell growth, differentiation, and survival but also on metabolism [16]. Except for the FGF11 subclass members so-called intracellular or nuclear FGFs (iFGF), the FGFs act as autocrine, paracrine, or endocrine factors and signal by binding to a fibroblast growth factor tyrosine kinase receptor (FGFR). Four different genes encode for seven isoforms of FGFRs: 1b, 1c, 2b, 2c, 3b, 3c, and 4.

The FGF19 sub-family and FGFs signaling

The members of the FGF19 subfamily of FGFs, also known as endocrine FGFs (eFGF) show an atypical structure. They lack the extracellular heparin-binding domain (HBD), which means a lower heparin-binding affinity and no capacity of being retained in the extracellular matrix, thus conferring on them the ability to enter the systemic circulation. The eFGFs require the dimerization of an FGFR (FGFR1c, 2c, 3c, and 4) with a co-receptor to active their signal transduction pathways [17,18]. This co-receptor is a klotho protein, alpha klotho for FGF23, and beta-klotho (KLB) for FGF19 and 21. Downstream of the FGFR-klotho, the intracellular cascade goes through the phosphorylation of FGFR substrate 2α (FRS2α) and the activation of Ras-MAPKs and PI3K-Akt kinases [8,18,19].

Globally the eFGFs have an essential role in maintaining metabolic homeostasis [20,21]. FGF23 participates in the crosstalk between bone and kidney, intestine, and parathyroid gland to maintain the body mineral balance, vitamin D3 homeostasis, and finally the bone health [20]. On the other hand, FGF19 (FGF15 in rodents) and FGF21 are involved in the maintenance of body weight and metabolic homeostasis by regulating glucose and lipid metabolism [20–22].

FGF21 and fasting

During the fasting state, several metabolic pathways are induced to maintain glycemia and energy homeostasis. Briefly, during the early fasting, hepatic glycogenolysis is the primary source of glucose. Later on, during long periods of fasting and essentially when most of the glycogen is depleted, hepatic gluconeogenesis and ketogenesis become the primary energy sources. In this situation, WAT undergoes lipolysis to supply fatty acids and glycerol to the liver. Several hormones play an essential role in regulating the metabolic adaptation to nutrient deprivation; molecules such as insulin, glucagon, leptin, catecholamines, and more recently, liver-derived FGF21 are well-known regulators of this metabolic state. FGF21 has been described as the missing link to fasting [23,24], and several authors have described its crosstalk with insulin and glucagon [25–27].

Several experiments have shown that, in mice, hepatic FGF21 expression is induced by 12 to 24 hours-fasting [28–30]. Then, FGF21 is secreted from hepatocytes and activates ketogenesis, gluconeogenesis, lipolysis, fatty acid oxidation (FAO), and globally the metabolic adaptation to fasting state [23]. In humans, no induction was reported during 72h of fasting [31].The induction of FGF21 by fasting is only detectable after 7 to 10 days of nutrient deprivation and correlates with weight loss and the utilization of fuel derived from tissue breakdown [30,32].

It is well-known that FGF21 is a hepatokine, but how FGF21 exerts its metabolic effects is, at some point, controversial [33]. It has been described that white and brown adipose tissues, heart and skeletal muscle, and the liver itself are FGF21 direct target organs/tissues 8,35]. Thus, FGF21 induces the peroxisome proliferator-activated receptor γ coactivator protein-1α (PGC-1α) that regulates the gluconeogenic genes and increases the expression of glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), carnitine palmitoyl transferase 1a (CPT1a) and the hydroxymethylglutaryl CoA synthase 2 (HMGCS2) [34–36].

Furthermore, a brain-liver axis to maintain glucose homeostasis during prolonged fasting has been described [37]. It has been demonstrated that FGF21 can cross the blood-brain barrier and acts directly on the nervous system to stimulate sympathetic nerve activity. FGFR and KLB, the FGF21 receptors, are expressed in several regions of the brain [38]. In this context, it has been

proposed that FGF21 would exert its effects by inducing the ERK1/2 phosphorylation on the hypothalamic neurons, thereby stimulating the expression of corticotropin-releasing hormone (CTRH) and finally activating the hypothalamic-pituitary-adrenal (HPA) axis [37,39]. This activation underlies the activation of CREB, which finally enhances the sympathetic nerve stimulation in BAT [39,40].

FGF21 expression during fasting state is driven mainly by the nuclear receptor peroxisome proliferator-activated receptor α (PPARa) [28–30,35,41,42] and the cyclic adenosine monophosphate (cAMP)-responsive element-binding protein H (CREBH, encoded by CREB3L3) [43,44]. The promoter of FGF21 contains a peroxisome proliferator responsive element (PPRE) for PPARa and a CREBH binding site (CHRE), indicating that FGF21 is under the dual control of both transcription factors [44–47]. Moreover, CREBH and PPARa are both cross-regulated by each other at the transcriptional level [48,49]. Finally, besides the role of CREBH in hepatic adaptation to energy starvation by direct regulation of gene expression, CREBH also acts as a PPARa coactivator participating in the recruitment of PGC-1α to the FGF21 promoter [50].

To define more precisely how CREBH activates the FGF21 expression, some recent publications have associated the expression of CREBH and finally FGF21 to a change in the histone acetylation profile of the CREBH gene. Concretely, experiments with the MS-275, a class I-specific histone deacetylase (HDAC) inhibitor caused an increase in the Histone H3 Lysine 18 acetylation

(H3K18ac) and promoted the hepatocyte nuclear factor 4 alpha (HNF-4α) recruitment in CREBH promoter. HNF-4a induces the expression of CREBH and finally, the FGF21 production [51]. On the other hand, the CREBH activity and, by extension, the FGF21 expression is also regulated by the endoplasmic reticulum-associated protein degradation (ERAD) machinery concretely through the Sel1L-HDR1 complex [52]. ERAD is responsible for recognizing and translocating protein for cytosolic proteasomal degradation. Its expression is induced in the postprandial condition upon mouse refeeding. In this context, an inverse correlation between the Sel1L-Hrd1 complex and Crebh-Fgf21 levels was described. The proposed mechanism is that Sel1-HDR complex drives the polyubiquitination, turnover, and reduced nuclear CREBH in mice [53].

Other proteins that regulate FGF21 expression and function under nutrient deprivation are SIRT1, an NAD+-dependent deacetylase, and Jumonji D3 (JMJD3), a histone demethylase. It is well-known that SIRT1 plays a critical role in mediating hepatic fasting responses through the deacetylation of different transcription factors or coactivators such as PGC1a [54–57]. Regarding FGF21 induction, it has been described that under fasting conditions, SIRT1 would be phosphorylated by cAMP-dependent protein kinase (PKA) and thus would let to the formation of a ternary complex with JMJD3 and PPARa [58]. This complex, apart from FGF21, would be responsible for inducing genes related to mitochondrial b-oxidation but no gluconeogenic genes [58].

Finally, it is worth to mention that FGF21 also controls the fasting-refeeding transition. Some authors described that during the refeeding period, there is an induction of Fgf21 expression in eWAT and repression of the fasting-induced Fgf21 expression in the liver [59]. Other authors, through liver-specific knockout mice, pointed out that the circulating FGF21 during this refeeding period would still come from the liver [24]. In humans, the induction of plasma levels of FGF21 under increases of glucose and insulin levels was also observed [60,61]. These data highlight the pleiotropic role of FGF21 in the maintenance of metabolic homeostasis and its tissue and nutritional-specific regulation.

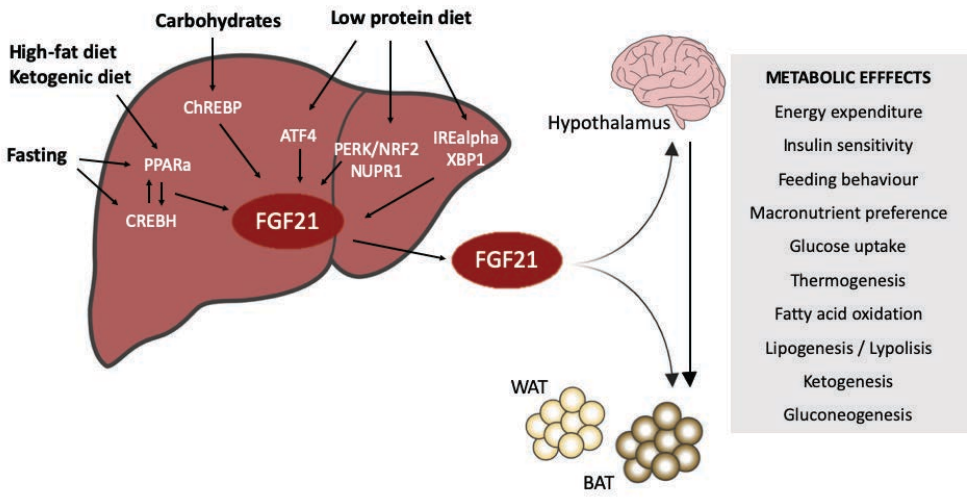


Figure I1. FGF21 induction by nutritional inputs. Low protein diet, carbohydrates intake, fat consumption, and fasting are nutritional signals that, through different transcription factors, induce the hepatic expression of FGF21. FGF21, as an endocrine factor, exerts its metabolic effects mainly in the adipose tissues directly or indirectly via a liver-hypothalamus axis. The metabolic effects of FGF21 look for restoring metabolic homeostasis by activating

different metabolic pathways and changing the macronutrient preferences and feeding behavior.

FGF21 and protein intake

Amino acid starvation initiates a signal transduction cascade called Amino Acid Response (AAR). The first step is the activation of the general control nonderepressible 2 (GNC2) kinase. GCN2 is an amino acid sensor that, once activated, phosphorylates, and inactivates the eukaryotic initiation factor 2 (eIF2a). Phosphorylation of eIF2a factor leads to a repression of the global translation except for some proteins that, under the ATF4 transcription factor, are produced to counteract the amino acid restriction [62]. ATF4 is part of the integrated stress response (ISR) in the liver and participates in the cellular response to different stressors, including amino acid deprivation.

The metabolic effects of a low protein intake include an increase in food intake and energy expenditure and an alteration in amino acid, lipid, and glucose metabolism [63,64]. Moreover, dietary amino acid composition and specific amino acid levels are also crucial in the metabolic response activated [63].

FGF21 is induced in mice by leucine, methionine/cysteine, and asparagine deprivation but also by low protein diets [65–68] as a part of the transcriptional program mediated by ATF4 [10,14,67,69]. In humans, protein or specific amino acids-reduced diets also

induces FGF21 levels [65,66,70,71]. Globally, FGF21 has been identified as a critical mediator in the metabolic response associated with amino acid or protein intake deficiency [63,66]. No induction of FGF21 was observed under caloric restriction if there is not protein reduction [72]. Under low protein intake, the induction of FGF21 mainly depends on GCN2/ATF4 [14] pathway, but GCN2-independent mechanisms have also been described. [73][74]. These alternative mechanisms would include PERK [75] or other stress-related proteins such as the liver-integrated stress response-driven nuclear protein 1 Nupr1 [65] or IREalpha-XBP1 [76]. PPARa signaling has also been implied in the induction of FGF21 under protein restriction [66].

It has been widely demonstrated that hepatic FGF21 is required for low protein diet-induced weight loss and increased energy expenditure [66]. It has been described that under amino acid-deficient diets o low-protein diets there is a reduction of de novo lipogenesis in the liver by inhibition of the fatty acid synthase (FASN), a reduction of lipogenic genes and an induction of lipolytic genes and finally an increased expression of thermogenic genes in BAT and WAT [10,67,77–80]. Although most of the authors described the adipocytes as the targets for FGF21 action under low-protein diets, a recent paper has included the CNS signaling as an intermediate step for the FGF21 metabolic response to low-protein diets [81]. Through the deletion of the KLB co-receptor, Hill et al. show that these mice are unable to switch on the metabolic response to a dietary protein restriction.

The real question is that in low-protein diets, caloric intake is usually achieved with an increment in the content of carbohydrates, and sometimes, it is difficult to distinguish between low protein diets or high carbohydrates-diets. As has been mentioned before, the expression of Frgf21 is sensitive to nutrient deficiency, and maximum serum levels of FGF21 are found in low protein and high carbohydrate intakes. In this way, it has been published that in rats fed with a low-protein, high-carbohydrate (LPHC) diet, the increase of serum levels of FGF21 correlates with an increase in UCP1, TBX1 (T-box transcription factor), and PRDM16 in perirenal adipose tissue (periWAT) [78]. This expression pattern, combined with the presence of multilocular adipocytes, suggested the occurrence of browning promoted by diet, a similar metabolic pattern observed with diets that are just defined as low-protein diets.

Instead of low protein-diets for long periods, Li et al. has described that periodic low-protein diets/high carbohydrate (pLPHC) shows similar metabolic benefits. pLPHC causes an increase in FGF21 levels, and induction of the thermogenic program and an obese-protected phenotype, despite an increased total energy intake. The question with this diet is that the improvement in insulin sensitivity was lost within 14 days of switching back to control diet among that the FGF21 induction correlates with Nupr1 overexpression suggesting a liver integrated stress response [82].

FGF21 and carbohydrates

The carbohydrate content of the diet can modulate the hepatic expression of *Fgf21* mainly through the activation of the carbohydrate responsive element-binding protein (ChREBP) and, therefore, its serum levels. Interestingly, both the deficient carbohydrate diet and the carbohydrate-rich diet can induce the expression of FGF21.

It was described that rats after 24h fasting, and then refeeding for 12h with a high carbohydrate diet (HCD) showed an increased *Fgf21* expression in the liver and FGF21 circulating levels compared with rats refeed with a fat diet [83]. In this case, the metabolic adaptations include induction of lipogenesis, glucose uptake and glucose metabolism in the liver, and a reduction of fatty acids uptake and fatty acid oxidation in the liver; and induction of lipogenesis, glucose uptake, and glucose metabolism, and lipolysis in WAT [83].

The same FGF21 induction was observed in mice fed an HC diet containing 77% of energy as dextrose, 0.5% as fat, and 22.5% as protein [84]. In mice, an excess of carbohydrate intake increases the expression levels of UCP1, FGF receptor 1c (FGFR1c), and KLB mRNA in BAT, suggesting increases in FGF21 sensitivity and energy expenditure.

In humans, overfeeding increases FGF21 levels. Interestingly, carbohydrate overfeeding but no fat led to markedly increased serum FGF21 levels in humans, and an acute response was found with a fructose load. It appears that the production of FGF21 is an attempt to maintain glucose homeostasis when there is a state of nutritional excess.

Finally, it has been described that in response to carbohydrate intake, the hepatic production of FGF21 suppresses the sugar preference through a mechanism that involves hypothalamic signaling [85].

FGF21 and fat consumption

The excess consumption of calorie-rich foods and sedentary lifestyle drives the actual global obesity epidemic and is the cause of disability around the world. The impact of fat consumption on FGF21 expression and signaling is not easy to explain due to the variety of fat structures included in diets. It is evident that depending on the kind of fat intake, the metabolic effects will be different. In general, the mRNA levels of FGF21 under fat intake is regulated by PPARa.

Differences in FGF21 expression has been observed between mice fed a corn-oil high-fat diet versus mice fed a PUFA-enriched high-fat diet. The animals under corn-oil overfeed for five weeks showed more FGF21 mRNA levels than those whose intake fish-derived long-chain polyunsaturated n-3 FA (PUFA) [86]. By contrast, in mice

fed an HFD for 16 weeks, no changes in FGF21 expression versus mice fed a low-fat diet were detected [84]. Significant differences were described in neonatal mice where hepatic FGF21 expression is induced for suckling, probably due to the milk composition and its high FA content [87]. In this case, the FGF21 secreted activates the thermogenic program in BAT. In humans, lipid infusion increases the circulating levels of FGF21 [88]. A lipid infusion or a high-fat diet overfeeding for five days induces FGF21 levels [89,90]], but under a lipid tolerance test, a decrease in FGF21 levels was described [91].

It has been described that butyrate and α-lipoic acid also regulate the levels of FGF21 in the liver. Butyrate is mainly produced by intestinal microbiota through fermentation processes and can regulate gene expression due to its activity as a histone deacetylase-3 (HDAC3) inhibitor [92]. Regarding α-lipoic acid, its dietary supplementation induces hepatic and plasma levels of FGF21 in vivo and in vitro [93,94] on a CREBH-dependent mechanism [43].

FGF21 in obesity: an impairment in FGF21 signaling

The excessive consumption of calories that exceed the adipose tissue storage capacity has been linked to low-grade inflammation, endoplasmic reticulum stress, and insulin resistance. These defects increase the risk of metabolic diseases as non-alcoholic liver steatohepatitis (NASH), type 2 diabetes, cardiovascular diseases,

and different forms of cancer. FGF21 is considered an anti-obesity hormone that circulates at variable levels and plays a role in mediating the physiological response to metabolic changes [95]. Some studies have shown that FGF21 metabolic benefits are mainly attributed to adipose tissue activity, where it induces thermogenic gene expression, oxygen consumption, and heat production.

Obesity is a state in which circulating levels of FGF21 are elevated. This increment in the FGF21 levels has been described in obese mice, in rhesus monkeys fed an HFD and in the serum of overweight/obese humans [96,97]. This induction is probably a response to overcome excess energy income and triglyceride accumulation. However, in this situation, endogenous FGF21 levels appear to be ineffective, whereas high pharmacological doses induce its effects, promoting weight loss, improving glucose tolerance, and lowering serum free fatty acids.

The Yip1 domain family, member six gene (YIPF6) was identified recently by Wang et al. [98] Results showed that YIPF6 controls the sorting of FGF21 into COPII vesicles during the development of obesity in mice. Mice with a YIPF6 mutation presented higher FGF21 levels than controls, increased lipolysis, energy expenditure, and thermogenesis. Other results suggested that YIPF6 protein levels are associated with fatty liver and obesity, and it is correlated inversely with FGF21 levels in serum. These results explain the differences between endogenous FGF21 dysfunction during obesity and pharmacological FGF21 effects [98].

Other mechanistic research identified that FGF21 resistance might be caused by a downregulation of its receptors resulting in compensatory FGF21 production. In this context, the published data suggest that the mRNA levels of FGF receptor subtype 1c (FGFR1), the one with the highest affinity for FGF21 were reduced in the liver, WAT, and pancreas islets of obese mice. Furthermore, KLB expression was also reduced in WAT and islets of obese mice. FGFRs are widely expressed, but KLB cofactor is present in only a small number of rodent tissues, notably adipose tissue, liver, and pancreas. In mice, with a total ablation of KLB no FGF21 activity was detected. In those animals, the KLB disruption entirely abrogates acute FGF21 signaling in adipose tissue and liver. In contrast, some new findings suggest that FGF21 activity is not mediated by the downregulation of KLB expression in WAT.

In an adipose tissue-specific β-klotho transgenic mouse, the maintenance of β-klotho protein expression in WAT does not alleviate the impairment in FGF21 signaling associated with obesity, indicating that the KLB expression downregulation may not be the primary mechanism contributing to impaired FGF21 signaling in WAT [99].

FGF21 is also elevated in other pathological conditions such as insulin resistance and liver diseases. In these cases, an impairment in FGF21 signaling has been described [100–102]. In humans, the resistance to FGF21 is not understood, and two possible

mechanisms have been proposed. A fibroblast activation protein (FAP) is a serine protease that cleaves and inactivates the FGF21has been described [103,104]. The presence of FAP reduces the ratio of active FGF21 to total FGF21 limiting its metabolic effects, even the high circulating levels detected. It has been described that patients with type 2 diabetes have elevated levels of circulating FAP compared to non-diabetic subjects thus suggesting that insulin resistance led to an inactivation of FGF21 and the attenuation of its beneficial effects [61,105].

On the other hand, it has been identified a common single-nucleotide polymorphism (SNP) in KLB gene (rs2608819) that is associated with a reduction in the levels of KLB in the adipose tissue a higher body mass index (BMI) in obese subjects [106][107]. However, KLB, FGFR1, and FGFR3 expression in the liver are increased in obese humans, which may increase the responsiveness of this tissue to FGF21.

FGF21 in humans

Many questions are still open regarding the metabolic role of FGF21 in humans, especially under non-pathological conditions. Divergent data between mice and humans has been detected involving the mechanisms that induce the FGF21 expression but also on its metabolic effects.

As has been mentioned before, neither short-term fasting nor ketonic diet increase FGF21 serum levels but in prolonged fasting (7-10 days). By contrast, in humans, FGF21 seems to be secreted under carbohydrate intake as a postprandial hormone produced under the insulin signal [60,61,89,108], protein intake [10,66] or exercise [109]. A positive correlation between insulin and FGF21 levels was described [96,106], and a genetic variant of FGF21 has been associated with an increased sugar intake [110].

Genetic studies in humans have associated some SNPs in and around the FGF21 gene with carbohydrates, protein, fat, and alcohol preferences [111–113] but also a specific KLB expression profile has been described in humans. Beyond the liver and the adipose tissue, in humans, KLB is also detected in breast, bone marrow, and pancreas [114]. These differences may explain, at least in part, the particular metabolic effects of FGF21 in humans.

Obesity and NAFLD

The excessive consumption of calorie-rich foods, together with a sedentary lifestyle, drive the actual global obesity pandemic. Obesity is the cause of several comorbidities, such as non-alcoholic fatty liver disease (NAFLD), which is the most common hepatic disease, affecting a quarter of the global adult population [1]. NAFLD is a chronic disease that begins with simple hepatic steatosis and can progress to non-alcoholic steatohepatitis (NASH) and fibrosis, causing severe damage, inflammation, and predisposition cirrhosis

[115,116]. The etiology of NAFLD is multifactorial and implies environmental, metabolic, genetic, and ethnic factors. Obesity and its metabolic disturbances, such as high serum TG levels, elevated systemic blood pressure, and hyperglycemia is considered a strong risk factor in the development of NAFLD [117].

NAFLD is characterized by an imbalance between FFA influx and export, and/or catabolism. The increased flux of free fatty acids into the liver are originated principally by lipolysis in adipose tissue (59%), then by de novo lipogenesis (26%) and diet (15%) [118].

High-fat diets induce obesity and insulin resistance; this energy overload and low energy expenditure are strongly related to fat deposition in the liver [117]. Adipose tissue contributes to NAFLD by sensing energy status, regulating the flux of free fatty acids arriving at the liver, and producing hormones and cytokines [117,119]. Nevertheless, during long-term caloric excess, white adipose tissue is expanded by hypertrophy of existing adipocytes and hyperplasia of the tissue, which increases the triglycerides storage capacity to protect other organs from ectopic fat accumulation and lipotoxicity, leading to an impairment of WAT function [119].

Overnutrition causes uncontrolled inflammatory responses in WAT, producing chronic inflammation, and inducing insulin resistance [119]. In this context, when the triglycerides storage capacity is overwhelmed, ectopic fat accumulation occurs in non-adipose tissues such as the liver, heart, and muscle [119]. This abnormal fat

deposition in the liver in the absence of excessive alcohol consumption is a characteristic feature of NAFLD. However, this condition is considered non-harmful, but it can progress into non-alcoholic steatohepatitis (NASH), which involves hepatic damage, fibrosis, and inflammation in the liver [117]. This altered condition in the liver, together with an altered endocrine function in WAT produce lipotoxic metabolic stress, low-grade inflammation, hypoxia, and fibrosis, contributing to adipocyte and hepatocyte dysfunction and insulin resistance [116].

Lipid induced hepatic insulin resistances leads to impaired insulin stimulation of hepatic synthesis. Impaired lipolysis can promote hepatic gluconeogenesis by an increase in hepatic acetlyl-CoA and pyruvate carboxylase (PC) activity, and promotes ectopic lipid storage in the liver [120].

Emerging evidence suggests an important role for other organs such as adipose tissue, muscle, and intestine in NAFLD development. Studies with animal models demonstrate that changes in dynamical lipid fluxes, rather than static triglycerides accumulation, determine whether simple steatosis will progress to NASH, thus indicating that NAFLD can be considered a systemic metabolic disorder [115].

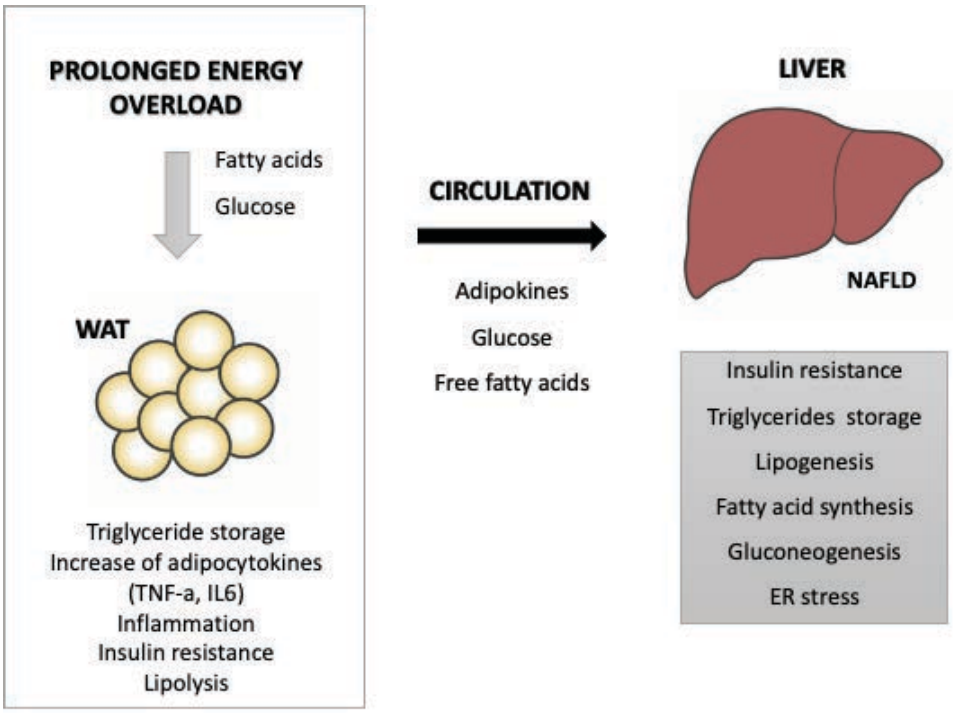


Figure I2. Prolonged overnutrition increases triglyceride storage in WAT, uncontrolled inflammatory responses leading to chronic inflammation, and inducing insulin resistance. Insulin resistance leads to impaired lipolysis, which promotes an increase free fatty acid flux to the liver and induction of gluconeogenesis by the increase in hepatic acetyl-CoA and pyruvate carboxylase activity. These mechanisms promote ectopic fat accumulation in the liver.

NAFLD and lipid metabolism

Adipose tissue lipolysis and de novo lipogenesis are both critical pathways involved in the pathogenesis of NAFLD. These pathways contribute to the increased flux of fatty acids to the liver. These mechanisms are controlled by insulin; however, NAFLD patients are usually insulin-resistant, leading to increased circulation of free fatty acids to the liver [117].

Some lipogenic genes had been described as essential regulators and possible targets in the treatment of lipid metabolism disturbances. The hepatic sterol regulatory element-binding protein (SREBPs) is a family of transcription factors involved in physiological processes as the biogenesis of cholesterol, fatty acids, and triglycerides [121]. It has been described that SREBPs participate in some pathological processes in the liver associated with the pathogenesis of NAFLD, NASH, hepatitis, and hepatic cancer.

SREBP-1c partly mediates insulin effects on hepatic lipogenesis. Insulin enhances SCAP/SREBP complex export to the Golgi and proteolytic processing of the SREBP-1c by decreasing the levels of Insig-1/2, thus stimulating the expression of SREBP-1c target genes [122,123]. Other documents provide evidence of SREBP-1c stimulating lipogenic enzymes and producing lipid accumulation related to insulin resistance [122].

AMPK, an energy sensor for cellular energy homeostasis, has been identified to inhibit SREBP cleavage and transcriptional activation. It was described that metformin stimulated AMPK activity, and it suppressed SREBP-1c cleavage leading to liver fat storage reduction [122]. Some of the causes contributing to SREBP stimulation are western diets, which are high sources of fat and fructose, both being identified as stimulators of SREBP1c expression and lead to hepatic lipid accumulation [116].

PPARs are ligand-activated transcription factor part of the nuclear receptor family. Three isotypes have been identified: PPARa, PPARb, and PPARy. Each isotype is activated by different ligand and has different tissue distributions and functions. However, they all control lipid and glucose metabolism, inflammation, and are involved in energy homeostasis. Because of its actions over lipid and glucose metabolism, PPARs are considered essential in the pathogenesis of NAFLD [117].

PPARy is active in the fed state and regulates fat deposition in WAT, and because of its actions in the liver, it has been considered a transcription factor related to the pathogenesis of NAFLD. In normal conditions, hepatic PPARy expression is deficient, but during hepatic steatosis, levels appear higher in mice; however, this effect is not found in humans. In rodents, PPARy contributes to transcriptomic and metabolic perturbations, as C3H mice, which do not express PPARy in the liver, appear to be resistant to HFD-induced steatosis [124]. These effects are due to PPARy in the upregulation in its target genes involved in lipogenesis such as *Acc1, Cd36, Fasn,* and *Scd1*; triglyceride synthesis: *Mogat1*; and lipid droplet formation: *Fsp27, Plin2* and *Cidea* [125,126].

A PPARy deficiency in adipose tissue induces a significative loss of this tissue and severe insulin resistance, causing fat deposition in the liver [127]. Also, it has been described that PPARy enhances insulin sensitivity by inducing adipokines, such as leptin, adiponectin, and FGF21 [128]. In this case, after PPARy induces

FGF21, it regulates reciprocally PPARγ activity by repressing its SUMOylation [117], causing an improvement in insulin sensitivity [128]. Also, it has been described that PPARγ stimulates thermogenesis in brown adipose tissue, which can contribute to this metabolic improvement.

Another abundant nuclear receptor in the liver is PPARα, which has an essential role in the regulation of lipid metabolism in this tissue. A study with a whole-body and hepatocyte-specific PPARα-deficient mice showed that PPARα deficiency aggravated liver steatohepatitis and hepatic inflammation upon HFD and methionine and choline-deficient diet feeding [129,130]. Mice with hepatocyte-specific deletion of this nuclear receptor presented spontaneous steatosis and liver damage [131]. Evidence supports the function of PPARα in the control of hepatic inflammation [132], and anti-fibrogenic effects [129,133]. The mechanisms by which PPARα is involved in this effect on the liver are by the downregulation of inflammatory genes as nuclear factor NF-kappa B, NFKB, signal transducer and activator of transcription (STAT), and activator protein-1 (AP-1), inflammatory transcription factors [117]. PPARs modulate transcription of genes involved in NAFLD-related functions in multiple organs and its role in inflammation. Therefore, PPARs appear as a relevant target to treat hepatic steatosis [117].

Some clinical models had reported curative effects on NASH with PPARα pharmacological activation [129,130]. In rodent models of methionine and choline deficiency diet-induced steatohepatitis, the

administration of the PPARa agonist Wy14643 decreases hepatic triglyceride levels, liver fibrosis, and inflammation [134]. Although PPAR agonists have had beneficial effects in rodents, their effectiveness in humans is limited. The administration of PPARs ligands in humans indicates that the PPARa activation reduces TG levels, whereas PPARy agonists improve insulin sensitivity and steatosis [117].

The cJun NH2-terminal kinase (JNK) is a stress signaling pathway that has been related to the HFD-induced insulin resistance and obesity [135]. Recent studies showed that activation of the JNK pathway represses PPARa, thus decreasing its target gene expression in the liver and causing repression in the fatty acid oxidation, ketogenesis, and promoting insulin resistance. This response is due to the increased expression of nuclear receptor corepressor 1 (Ncor1) and nuclear receptor-interacting protein 1 (Nrip1) co-repressors via AP-1 binding sites in their promoters. Also, JNK deficiency in HFD-fed mice leads to FGF21 increased expression and elevated plasma levels, which improves systemic metabolism [116,135]. A disturbance in the hepatic PPARa-FGF21 hormone axis suppresses the metabolic effects of JNK deficiency, indicating that FGF21 has a vital role in the JNK signaling in the liver [135].

Several studies have shown that FGF21 levels in serum are elevated in subjects with NAFLD, and its levels correlate with the hepatic fat content. Thus, FGF21 has been suggested as a potential

diagnostic marker of NAFLD as a marker indicating liver damage [136]. Furthermore, it has been reported that FGF21 can exert effects on reducing fat content in the liver and decreasing fibrosis and inflammation [4,5]. However, the functional role and molecular mechanism of FGF21 induction in obesity and NAFLD are still unclear.

Tunicamycin and endoplasmic reticulum stress

Tunicamycin causes lipid accumulation in the liver by the induction of ER stress and inhibiting hepatic gluconeogenesis [137,138]. Tunicamycin inhibits the phosphorylation of protein kinase B (Akt), which plays an essential role in insulin sensitivity, glucose, and lipid metabolism [139].

Perturbations of ER homeostasis produce an excessive accumulation of misfolded proteins in the ER lumen, triggering the uncoupled protein response UPR [140]. UPR is a cellular response mechanism activated as an adaptative program to counteract the misfolded protein accumulation in the ER. Three ER transmembrane sensors regulate this mechanism: Inositol-requiring 1a (IREα), activating transcription factor 6α (ATF6), and PKR-like kinase (PERK) [140]. IREα is the most conserved sensor of the ER stress possessing both protein Ser/Thr kinase and endoribonuclease (RNase) activities.

Under ER stress conditions, IRE1 is activated through trans-autophosphorylation and dimerization/oligomerization, and catalyzing catalyzes the removal of a 26-nucleotide sequence from an intron of in the mRNA intron that encodes for the transcription factor X-box binding protein 1 (XBP1). XBP1s, an active spliced form of XBP1, initiates then a critical part of the UPR program [76]. Besides, PERK phosphorylation of eukaryotic translation factor 2a (Eif2α) leads to simultaneous induction of the activating transcription factor 4 (ATF4), which participates in various adaptative responses to ER stress downstream of IRE1a and PERK.

The UPR aims to decrease ER stress and promote cell survival; however, when ER stress is severe and exceeds the capacity of the UPR, it has pathological consequences, including cell death, insulin resistance, hepatic lipid accumulation and inflammation [141,142]. ATF4, ATF6, and XBP1-sp activate transcription of CCAAT enhancer-protein homologous protein (CHOP) by binding to the appropriate promoter region. CHOP is a key factor involved in ER stress-mediated apoptosis [15].

Many metabolic disturbances are linked to liver ER stress. In these conditions, PERK and XBP1 stimulate the expression of ATF4 signaling leading to FGF21 upregulation. FGF21 expression is linked to hepatosteatosis, liver damage obesity, and oxidative stress, thus considering FGF21 a possible biomarker of liver dysfunction [143]. Mitochondrial disfunction also triggers ER stress and stimulates the expression of FGF21. Also, ER stress has been

linked to KLB stimulation via the ATF4. This mechanism might improve the auto/paracrine signaling of secreted FGF21 and thus provide a positive feedback mechanism to alleviate stress-induced hepatic disturbances [143,144].

Materials and Methods

Protein extraction

Tissues extracts from liver, subcutaneous adipose tissue (scWAT), brown adipose tissue (BAT) and hypothalamus were homogenized with Polytron Ultra-turrax with low intensity in RIPA buffer and centrifuged at 12000 x g for 15 min at 4ºC. RIPA buffer was supplemented with a mixture of protease inhibitors (Ref. P8340, Sigma-Aldrich, USA), and a phosphatase inhibitor cocktail 3 (Ref. P0044, Sigma-Aldrich, USA). The supernatant was collected and frozen at -80ºC for further analysis. Protein concentration was determined with the Bio-Rad Protein Assay Dye Reagent Concentrate (Ref. 5000006, Bio Rad, USA) following the manufacturer instructions. The standard curve was prepared with different concentration of Bovine serum albumin (BSA) diluted in RIPA buffer.

- RIPA buffer: Tris HCL 10mM pH 7; Triton X-100 1%; NaCl 150m; EDTA 5mM.

Western blotting analysis

Protein extracts were resolved by 8-10% SDS-polyacrylamide gel electrophoresis and transferred into a PVDF membrane (Milipore) with iBlot 2 Dry Blotting System (ThermoFisher Scientific) program 0 (20V 1 minute, 23V 4 minutes, 25V 5minutes).

Membranes were blocked for 1h at room temperature in a blocking solution and then incubated with the primary antibody overnight at 4ºC. To eliminate the unbound antibody, the membranes were washed three times for 10min each with the washing solution. Finally, membranes were incubated with the secondary antibody for 2h at room temperature.

The secondary antibody was also diluted in blocking solution. The excess of the secondary antibody was eliminated by washing the membranes three times for 10 minutes with a washing solution. The secondary antibodies used were linked to horseradish peroxidase enzyme. To develop where primary and secondary antibody were bound, the membranes were incubated with a substrate for peroxidase and chemiluminescence´s enhancer for 1min and immediately scanned with c-Digit LICOR. Quantification was made with densitometry Image J software.

Antibodies were diluted according to the manufacturer´s instructions in blocking solution as indicated:

Antibody	Antibody Dilution	Blocking solution	Washing solution	Secondary antibody
P-S6 Ribosomal Protein Rabbit Antibody	1:5000	TBS 1X 0.05%TWEEN 3%BSA	TBS1x 0.05% TWEEN	Na934vs ECL Peroxidase anti-rabbit antibody Lot. 9599347
SG Ribosomal Protein Rabbit Antibody	1:5000	TBS 1X 0.05%TWEEN 3%BSA	TBS 1x 0.05%TWEEN	Na934vs ECL Peroxidase anti-rabbit antibody Lot. 9599347

TBS 1X
25 mM Tris
150 mM NaCl
2 mM KCl
pH 7.4

RNA isolation

Total RNA was isolated from frozen tissues using TRI Reagent™ Solution (AM9738, Thermo Fisher Scientific, Waltham, USA) and homogenized with the Polytron at a low intensity for 10 seconds. The samples were incubated at room temperature for 5 minutes and centrifuged at 12000 x g for 10 minutes at 4ºC, and the supernatant transferred to a fresh tube. Next, 1 ml of Isopropanol was added to the sample tube, vortexed for 5-10 seconds, and incubated by 5-10 min. Then, the samples were centrifuged at 12000 x g for 8 min at 4ºC, and the supernatant was discarded. Afterward, 1 ml of 75% ethanol is added to the tubes and then centrifuged at 10000 x g for 5 min; then, the ethanol was removed, and the RNA pellet was air-dried.

RNA was dissolved in protease-free water, and the concentration and purity were measured in the microvolume spectrophotometer NanoDrop-1000 (NanoDrop Technologies, Inc. Thermo Scientific). Absorbance at 260, 230, and 280 nm was measured, and A260/A280 and A260/A230 ratios were calculated to evaluate the concentration and purity of the samples.

To remove the traces of genomic DNA the DNaseI treatment was used following the manufacturer instructions (K2981, Thermo Fisher Scientific, Waltham, USA).

Relative quantitative RT-PCR

cDNA was synthesized from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific, Waltham, USA). Relative mRNA levels were analyzed by quantitative PCR (qPCR) using SYBR™ Select Master Mix for CFX (4472942, ThermoFisher Scientific, Waltham, USA) or Taqman Gene Expression Master Mix supplied by Applied Biosystems (ThermoFisher Scientific). Each sample was measured by duplicate, using 18S and B2M as housekeeping genes. Results were obtained by the relative standard curve method, and values were referred to the HFD group. Results were obtained by a standard curve method and then expressed as fold induction compared with the experimental control.

DNA oligonucleotides (primers and probes)

DNA oligos were synthesized by Sigma-Aldrich with ultra-high base coupling efficiency technology, combined with optimized cartridge purification and 100% quality control by mass spectrometry.

	Gene	Sequence/ref
Sybr Green	Glut1	F-5´-GCCCCCAGAAGGTTATTGA-3´ R-5´-CGTGGTGAGTGTGGTGGAT-3´
Sybr GreeN	Glut4	F-5´-GTGACTGGAACACTGGTCCTA-3´ R-5´-CCAGCCAGTTGCATTGTAG
Sybr Green	Srebp1c	F-5´-GGAGCCATGGATTGCACATT-3´ R-5´-GGCCCGGGAAGTCACTGT-3´
Sybr Green	Cd36	F-5´- GGAGCAACTGGTGGATGGTT-3´ R-5´- CTACGTGGCCCGGTTCTAAT-3´
Sybr Green	Fasn	F-5´-GCTGCGCGGAAACTTCAGGAAAT-3´ R-5´-AGAGACGTGTCACTCCTGGACTT-3´
Sybr Green	Klb	F-5´- GAGAACGGCTGGTTCACAGA-3´ R-5´- GGCCGTATAACCAAACACGC-3´
Sybr Green	Erg1	F-5´-GCCGAGCGAACAACCCTAT-3´ R-5´-ATAACTCGTCTCCACCATCGC-3´
Sybr Green	Ucp1	F-5´-CCCGCTGGACACTGCC-3´ R-5´-ACCTAATGGTACTGGAAGCCTGG-3´
Sybr Green	Prmd16	F-5´-CAGCACGGTGAAGCCATT-3´ R-5´-GCGTGCATCCGCTTGTG-3´
Sybr Green	B2m	F-5´-TCACATGTCTCGGATCCCAGTAGA-3´ R-5´-ACTGATACATACGCCTGCAGAGTT-3´
Taqman	Fgf21	Mm00840165_g1
Taqman	18s	X03205.1

Animal experimentation

Mice housing

Animals were housed at the University of Barcelona Animal Facility of Farmacy Campus. Mice were kept in a room with controlled temperature (22 ± 1ºC) on a 12/12h light/dark cycle and were provided free access to tap water and chow diet before the experiments.

Experiments were performed with C57BL/6J male mice aged 4-6 weeks or 10 weeks old, depending on the experimental approach.

Mice strains

Mouse	Nomenclature	Origin
$Fgf21^{loxP}$	B6.129S6(SJL)-$Fgf21^{tm1.2Djm/J}$	The Jackson Laboratory
LFgf21KO	$FGF21^{fl/fl;\ Albumin\text{-}Cre}$	Dr. Haro and Dr. Marrero Laboratory

Mice interventions were performed with the approval of the animal experimentation ethics committee of the University of Barcelona.

Fgf21 liver specific knockout mouse colony management

To generate the Fgf21 liver-specific knockout mice (LFgf21KO), $Fgf21^{loxP}$ mice ($Fgf21^{tm1.2Djm/J}$) that have Fgf21 gene flanked by two loxP sites were crossed with Albumin-cre (Tg(Alb1-cre)1Dir7J) mice. The offspring of these mice express the CRE recombinase enzyme under the control of the albumin promoter element, thus letting liver-specific gene deletions.

Genomic DNA extraction

To extract DNA samples, 2mm of mice tail were snipped and stored in a microtube. Then, 500 ul of tail buffer (supplemented with proteinase K) was added to the snipped tails in each tube and were incubated at 55ºC overnight until tails are dissolved. Once tails are degraded, they were centrifuged at 1400 rpm for 10 min at room temperature and the supernatant was transferred to a new tube and added 500 ul of isopropanol mixing well.

Then, the tubes were centrifuged at 14000 rpm for 5 min at 4ºC. The supernatant was discarded, and the pellet was washed with 70% ethanol and again centrifuged at 14000 rpm for 5 min at 4ºC. Ethanol was discarded, and pellet air-dried with the tube opened at room temperature. Once dried the pellet was resuspended with 30 or 50 ul of TE pH 8. Finally, each sample was quantified to check DNA integrity and then loaded into a 1% or 3% agarose gel.

The solutions and concentrations used for each buffer are described below:

- Tail buffer: Tris-HCL 20 mM pH8; EDTA 5mM pH 8; SDS 0.5%; NaCl 200 mM.

- TE pH 8: Tris-HCL 10 mM pH 8; EDTA 1 mM.

PCR detection of Cre and LoxP

Detection of Cre and LoxP was made by a PCR using genomic DNA extracted from mice tails. CRE PCR product is around 100bp. Loxp PCR product is around 450bp for homozygous mutants, a single band of 319bp indicates a wild type mouse and both bands are specifying a heterozygous mutant mouse. All samples were loaded in a 3% agarose gel.

Animal interventions

Tunicamycin treatment

10 weeks old male mice C57Bl6/J LoxP (n=20) and LFKO (n=20) were randomly divided into two experimental groups: 1.- control group treated with vehicle (PBS) and 2.- group treated with TM (1mg / kg). 24 hours after TM administration (intraperitoneal injection), animals were slaughtered by employing cervical dislocation and the tissues were stored at -80°C for future analysis.

HFD-LP nutritional intervention treatment

Six-week-old male C57BL/6J mice (n=29) were randomly divided into a high-fat diet group (HFD) (n=14) or a high-fat low-protein diet group (HFD-LP) (n=15) for 10 weeks. Animals were housed in a temperature-controlled room (22 ± 1ºC) on a 12/12h light/dark cycle and were provided free access to tap water before the experiments. After 10-week of nutritional intervention, animals were euthanized. Blood was extracted by

intracardiac puncture, and serum was obtained by centrifugation (1500 rpm, 20 min), subcutaneous WAT (scWAT), BAT, hypothalamus and liver were isolated, immediately snap-frozen and stored at −80ºC for future analysis.

Mice were distributed 3-4 per cage, and during the nutritional intervention, body weight and food intake were measured twice per week. Food intake was recorded, assuming that each animal had the same intake.

Diet composition

Both diets were designed to be isocaloric by equally different protein and carbohydrate content while keeping fat constant. HFD: 45% fat, 35% carbohydrate, 20% protein, (D12451, Research Diets); HFD-LP: 45% fat, 50% carbohydrate, 5% protein (D14100702, Research Diets).

Fasting experiment

10-week-old male C57BL6/J mice with FGF21-specific liver depletion (LFKO) (n=16) and their respective control model (LoxP) (n=14) underwent 17h fasting. The animals were sacrificed together with mice fed ad libitum, and gene expression analysis was performed using qPCR of the main genes involved in the metabolic response to fasting.

Insulin and glucose tolerance test

Mice were fasted for 6 h in the morning and then injected intraperitoneally (i.p.) with 1.5 g of glucose (Sigma)/kg mouse (GTT) or 0.5 UI of insulin solution (Sigma)/kg mouse (ITT). Blood samples were collected from the

tail vein, and glucose levels were measured using a glucometer (Glucocard SM, Menarini, Florence, Italy) prior to the i.p. injection and at 30, 60, and 120 min postinjection.

Histological analysis

A piece of liver of each animal was fixed in 10% formalin (Sigma) and embedded in paraffin. Afterward 4 μm-thick sections were cut and stained with hematoxylin and eosin (H&E). Images were acquired using a Digital Upright Microscope BA310 Digital and a Moticam 2500 camera. The selection of the test objects was performed according to color and choosing the same limits for binarization for all images. At least four pictures from different regions of each cut were taken.

Triglycerides quantification

A small piece of liver of each animal was homogenized with a Dounce in 1 ml solution containing 5% Nonidet P 40 Substitute (Sigma-Aldrich, 74385) and water, slowly heated to 90°C in a water bath for 4 min until Nonidet P40 became cloudy, and then gradually cooled down to room temperature. Liver lysates were repeated by heating the tissue one more time and centrifuged at 4°C for 2 min at 12,000*g*. Triglyceride content was analyzed by Triglyceride Quantification Kit (Sigma Aldrich, MAK266) according to the manufacturer's protocol.

Hepatic transaminases quantification

Aspartate aminotransferase (AST) was measured in the liver of mice. 50 mg of tissue was homogenized in 200 ul of ice-cold AST Buffer. Samples

were centrifugated at 13,000 x g for 10 minutes to remove insoluble material. AST content was measured with the AST Activity Assay Kit (Sigma-Aldrich, MAK055) according to the procedure recommended by the manufacturer. To measure alanine aminotransferase (ALT), 50 mg of liver was homogenized with 200 ul of ALT Buffer. Then, samples were centrifuged at 15, 000 x g for 10 minutes to remove insoluble materials. Then, AST content was quantified using AST Activity Assay Kit (Sigma-Aldrich, MAK052) according to the procedure suggested by the manufacturer. In both assays, AST and ALT, enzymatic activity was measured by colorimetry (450 nm).

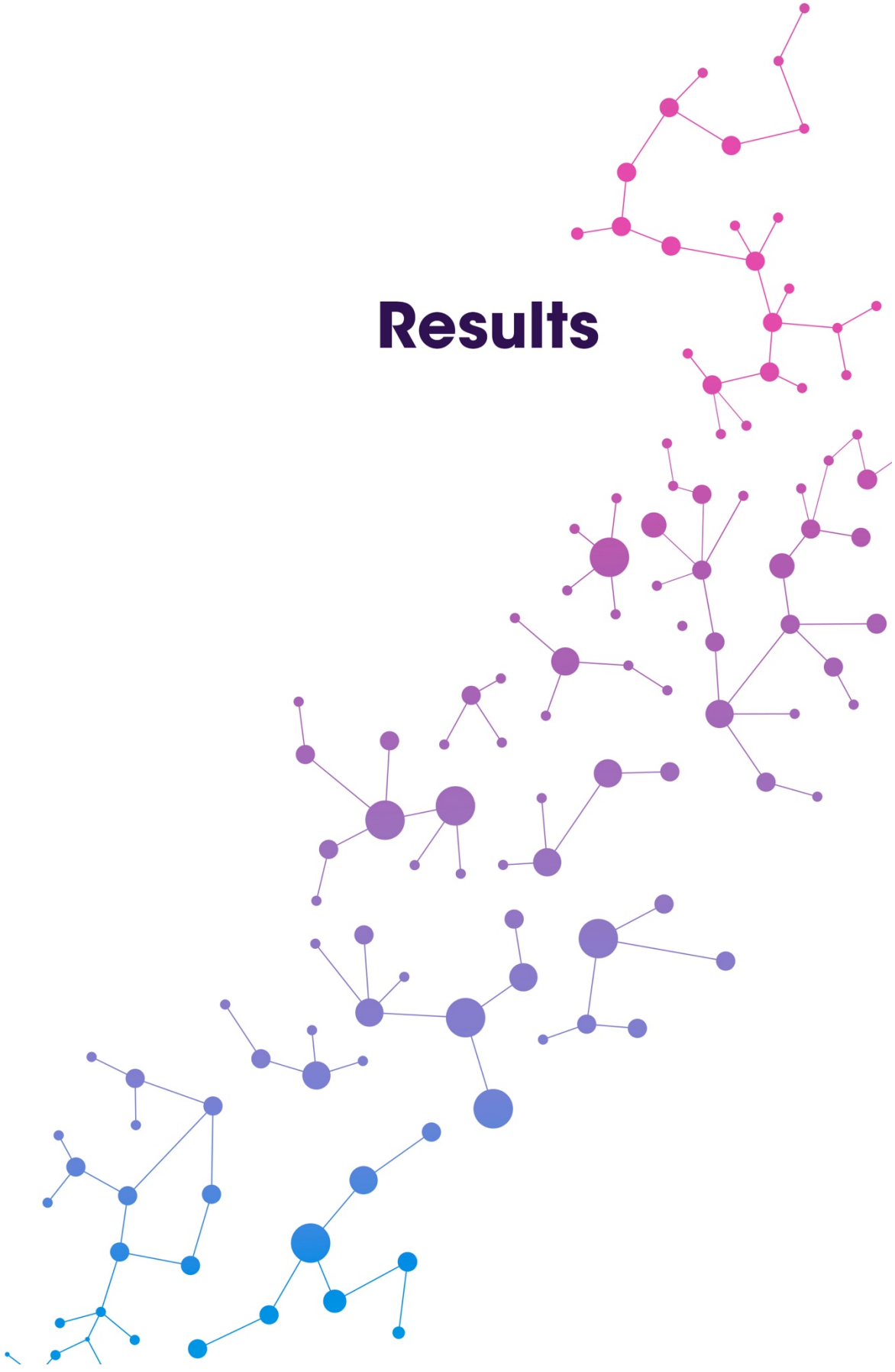
Immunoassay determination of FGF21

Concentrations of FGF21 in serum were determined in mice with an ELISA according to the procedure recommended by the manufacturer (EZRMFGF21-26K, Rat/Mouse FGF-21 ELISA kit, Merck Millipore). The minimal detectable concentration of FGF21 with this assay was 49.4 pg/ml.

Quantification and statistical analysis

GraphPad Prism version 8.02 (GraphPad, San Diego, USA) was used to perform the statistical analyses. Two-way ANOVA was carried out to compare multiple groups, and then Sidak´s adjustment was run. In all cases, p-value < 0.05 was considered statistically significant. All data are expressed as the mean ± SEM.

Results



Chapter 1

Role of FGF21 in the metabolic response to acute hepatic steatosis caused by tunicamycin administration

ER stress maintains energetic homeostasis by triggering the dephosphorylation and nuclear entry of CREB regulated transcription coactivator 2 (CRTC2 or TORC2) and its association with ATF6, leading to the induction of UPR control genes. ATF6 reduces the hepatic glucose output by disturbing the CREB-CRTC2 communication and thereby impeding CRTC2 occupancy over gluconeogenic genes [145].

FGF21 has been identified as a protein capable of reducing triglyceride storage in the liver [140] and restoring lipid homeostasis when there is metabolic stress.

TM is a drug that induces ER stress and hepatic steatosis. The molecular mechanisms underlying these effects are diverse. Firstly, TM produces an accumulation of unfolded or misfolded proteins by preventing the first step in the protein biosynthesis of N-linked glycans. This accumulation of misfolded proteins in the ER causes ER stress and activates the UPR [141]. Moreover, TM administration leads to lipid accumulation in the liver by stimulating lipogenic gene expression, inhibition of fatty acid oxidation, and the suppression of apolipoprotein B100 secretion [146] [137]. Thus, lipid storage in the liver occurs due to an impairment in the balance between de novo

lipogenesis, hepatic availability of free fatty acids, and hepatic lipid export by lipoproteins [147].

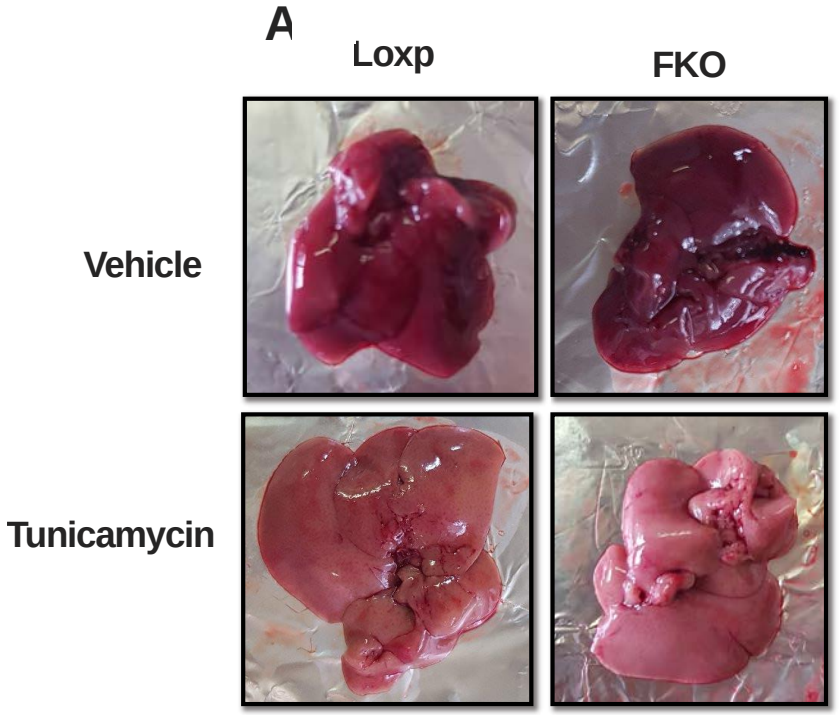
Finally, the ER stress caused by TM blocks the gluconeogenesis pathway in the liver. It has been demonstrated that the ER stress-induced downregulation of PEPCK and G6Pase expression is mediated by ATF6 overexpression [148]. Other authors also showed that TM inhibits gluconeogenesis by inhibiting the IL-6-dependent phosphorylation of the signal transducer and activator of transcription 3 (STAT3). The p- STAT3 interacts with the promoter region of the G6Pase gene and suppresses its expression, thus inhibiting the gluconeogenesis [149].

Considering that TM induces liver steatosis and FGF21 restores lipid homeostasis, we asked about the role of FGF21 in front of the metabolic and ER stress-induced by TM administration was. We analyzed the response of liver specific FGF21-knockout (LFKO) mice, and control mice (LoxP) treated with TM to determine hepatic FGF21 effects over lipid metabolism and ER stress in the liver.

The increment of triglycerides storage in the liver caused by TM does not depend on the hepatic FGF21

After 24h of TM Treatment, livers from LoxP and LFKO mice showed a more steatotic appearance in comparison with LoxP and LFKO mice treated with vehicle (Figure R1A). Data revealed that both LoxP and LFKO mice after the TM injection accumulated more TG in the

liver compared with their littermates injected with the vehicle. No differences were observed between TM-treated groups, indicating that the TG storage in the liver after 24h of TM injection does not depend on the presence of hepatic FGF21 (Figure R1B).



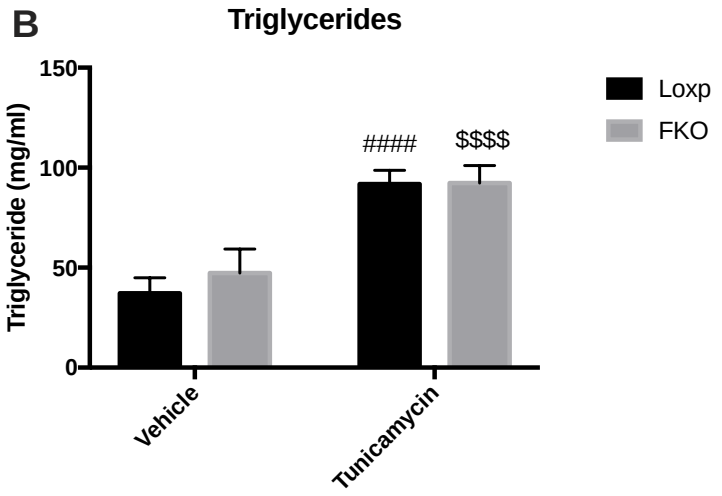


Figure R1. Liver image after sacrifice and triglyceride content in livers of LoxP and LFKO mice after 24h of vehicle or TM administration. (A) Liver images were taken immediately after liver extraction, pictures represent LoxP or LFKO mice injected with TM or vehicle after 24h. (B) Triglyceride quantification in livers of LoxP and LFKO mice. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs LoxP Vehicle. $p<0.05; $$p<0.01; $$$p<0.001 vs LFKO Vehicle.

Hepatic FGF21 is induced by ER stress and contributes to the UPR activation

As a mechanism to counteract misfolded proteins in the ER the UPR is activated [150]. ATF4 is a transcription factor involved in the UPR that stimulates the expression of genes located under the PERK arm of the UPR [151]. *Fgf21* is an ATF4 target gene and it has been documented that FGF21 levels are increased by ATF4 during ER stress [14,117,152].

Our results showed that Fgf21 expression was markedly increased in the liver after 24h of TM administration in LoxP mice (Figure R2).

Fgf21 mRNA was not detected nor in control groups (treated with vehicle) nor in FKO mice treated with TM as expected.

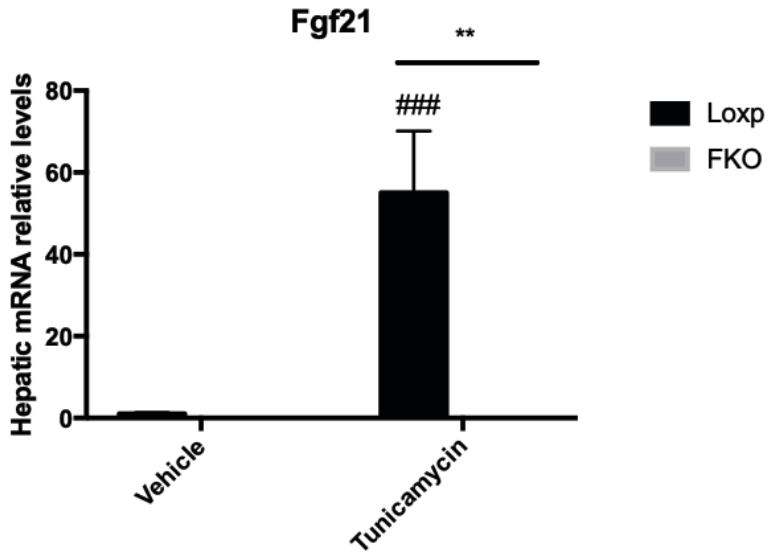
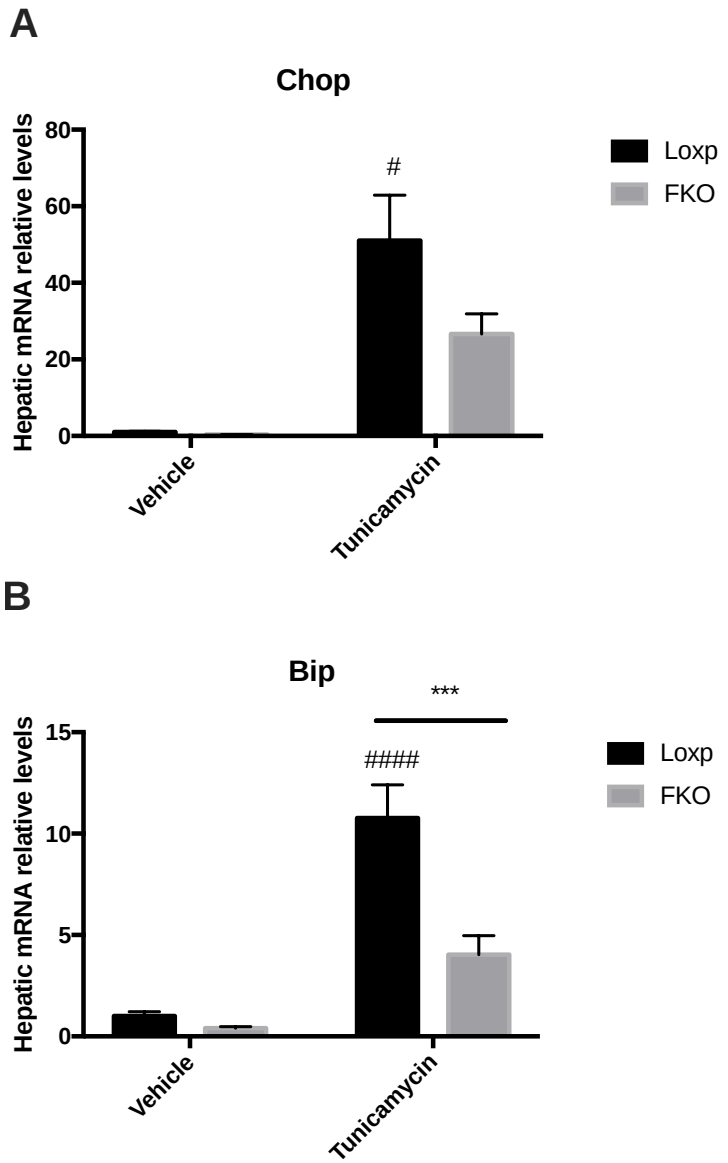


Figure R2. Gene expression of *Fgf21* in the liver of LoxP and LFKO mice treated with TM or vehicle. Average represented in graphs. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs LoxP Vehicle. *p<0.05; **p<0.01; ***p<0.001 vs LoxP Tunicamycin.

When unfolded proteins accumulate in the ER, resident chaperones begin to release transmembrane ER proteins involved in the UPR to decrease cell stress [153]. Part of the UPR is mediated by *Bip*, *Chop* and *Xbp1c-s*.

Our results showed that the expression of Chop, Bip, and Xbp1c-s was induced after TM injection in LoxP mice compared to vehicle-treated mice (Figure R3A, 3B, and 3C). In the case of Chop and Bip this induction is impaired in LFKO mice, thus indicating that Fgf21 is required for the full activation of the UPR after the TM administration.

By contrast, the induction of Xbp1c-s by TM treatment is independent of the Fgf21 as both TM-treated groups showed the same increase in the mRNA levels of this protein after the TM injection.



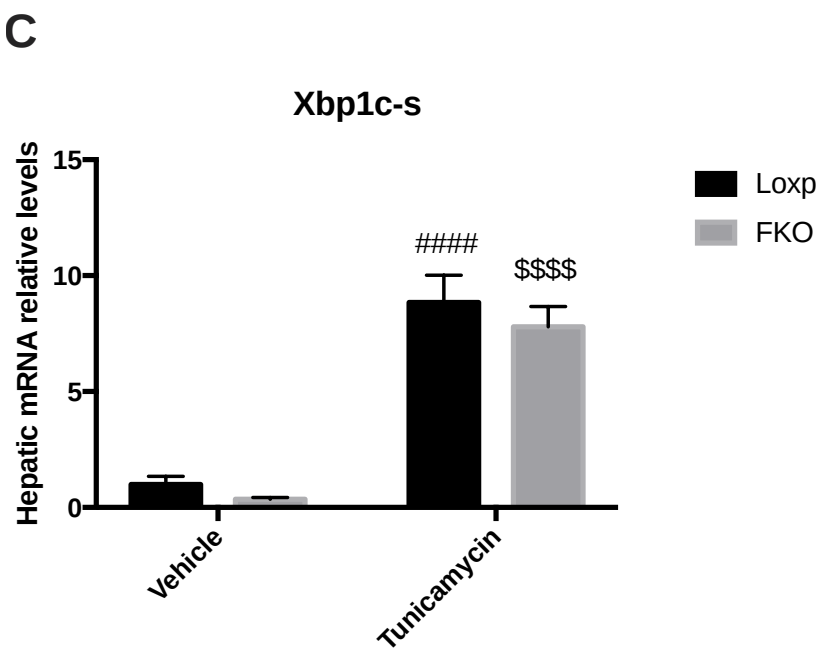
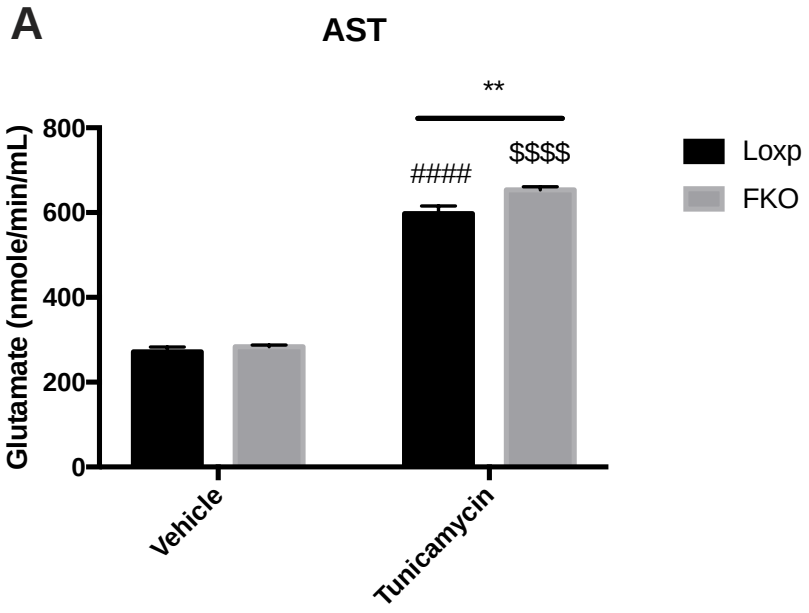


Figure R3. Gene expression of ER stress markers and mediators of the UPR in the liver of LFKO and LoxP mice treated with vehicle or TM. mRNA levels of: C/EBP homologous protein (*Chop*) (A); Binding immunoglobulin protein (*Bip*) (B); and Xbox-binding protein 1c (*Xbp1c*) (C). Average represented in graphs. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs LoxP Vehicle. $p<0.05; $$p<0.01; $$$p<0.001 vs LFKO Vehicle. *p<0.05; **p<0.01; ***p<0.001 vs LoxP Tunicamycin.

Hepatic FGF21 levels are related with hepatic transaminases levels

Hepatocellular damage triggered by ER stress and liver steatosis causes the release of the liver transaminases: aspartate aminotransferase (AST) and alanine aminotransferase (ALT). AST and ALT are key enzymes for liver function and are used as markers of liver damage when their hepatic and/or circulating levels increased [154].

FGF21 levels have been associated with transaminases levels in serum [32]. We found that hepatic AST levels were induced in both LoxP and LFKO mice treated with tunicamycin. However, the LFKO mice presented higher levels of AST than the LoxP mice (Figure R4A). By contrast, ALT levels were not induced after the TM treatment in LoxP mice, but yes, in LFKO mice. LFKO mice presented significantly higher AST levels, indicating that FGF21 is contributing to liver function and is related to transaminases levels (Figure R4B). Altogether these results showed that the liver damage caused by TM is ameliorated by hepatic FGF21, thus indicating that FGF21 may exert a protective effect in the liver.



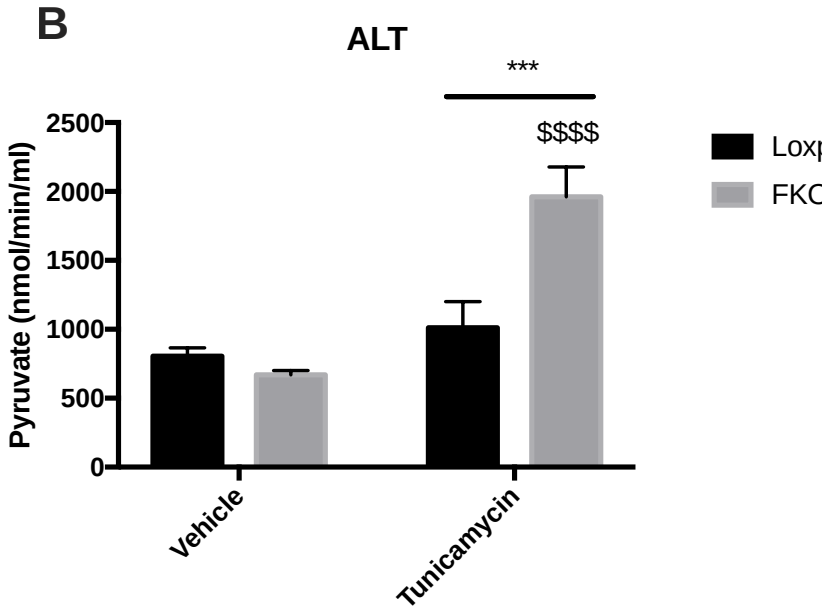


Figure R4. Hepatic transaminases levels in liver of LoxP and LFKO mice treated with TM or vehicle. Aspartate aminotransferase (AST) (A) and alanine aminotransferase (ALT) (B) quantification in liver. Average represented in graphs. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs LoxP Vehicle. $p<0.05; $$p<0.01; $$$p<0.001 vs LFKO Vehicle. *p<0.05; **p<0.01; ***p<0.001 vs LoxP Tunicamycin.

Genes involved in *de novo* lipogenesis are repressed in the liver after TM injection in a FGF21-CREBH independent way

Previous results showed that TM induced hepatic steatosis and TG accumulation (Figure R1B). Previously published data suggest that the FGF21-CREBH axis could induce hepatic steatosis by upregulating genes involved in fatty acid synthesis [155]. To evaluate the FGF21-CREBH axis' role, the mRNA levels of *Crebh* in control and TM-treated mice were analyzed. As shown in Figure R5, the expression of *Crebh* in LFKO control mice was considerably

lower than in LoxP control mice. After 24h of TM injection, *Crebh* mRNA levels were modified in livers of neither LoxP nor LFKO mice (Figure R5), thus suggesting that *Crebh* is not mediating the lipid metabolic response the acute stress caused by TM.

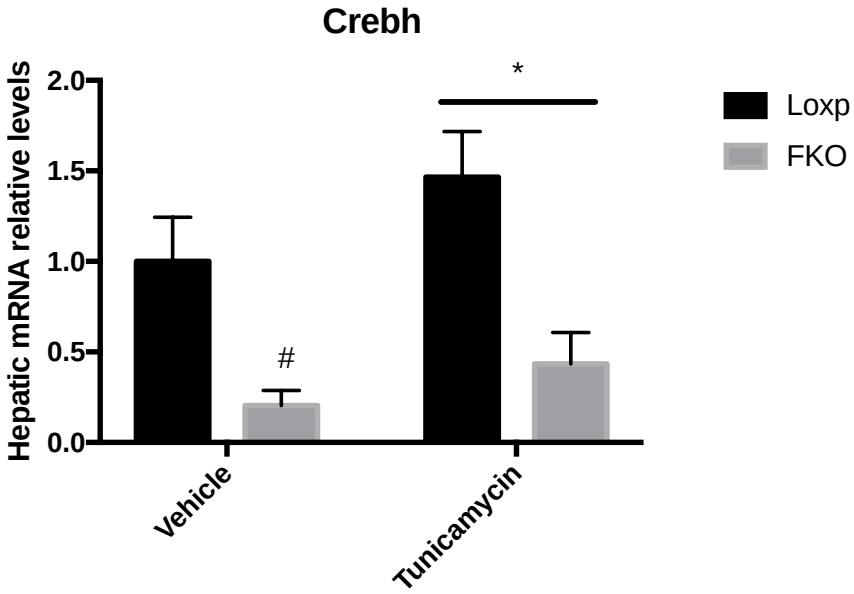


Figure R5. mRNA expression analysis of *Crebh*. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs LoxP Vehicle. *p<0.05; **p<0.01; ***p<0.001 vs LoxP Tunicamycin.

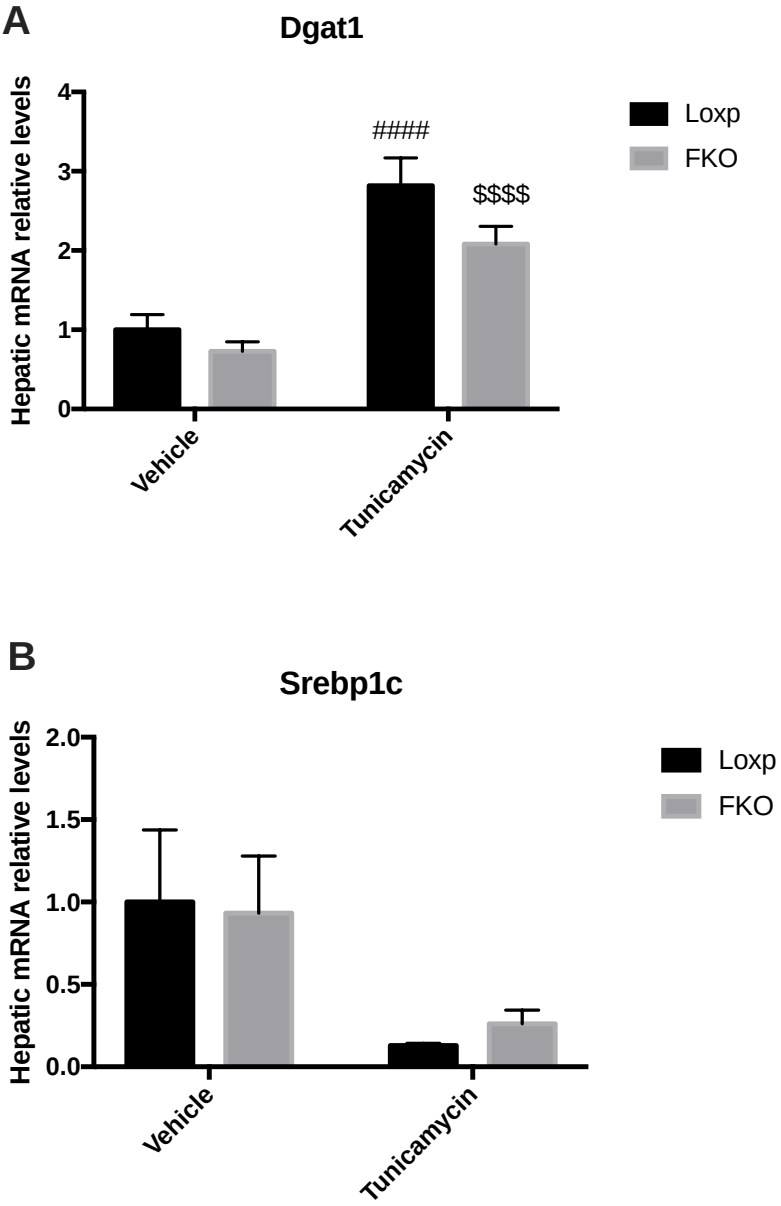
To evaluate the metabolic profile of TM-treated mice and the role of FGF21 on the metabolic effects of TM, the expression of genes related to fatty acid synthesis *diacylglycerol O-acyltransferase 1 (Dgat1), Fasn* and *Srebp1c* were measured in the liver of LoxP and LFKO mice after 24h of TM or vehicle injection.

DGAT1 is an enzyme that contributes to TAG synthesis on both sides of the ER membrane by esterifying pre-formed fatty acids to

glycerol [156]. The mRNA relative levels of *Dgat1* was markedly induced in LoxP and LFKO TM-treated mice without changes between groups (Figure R6A).

SREBP1c is a transcription factor that regulates the expression of lipogenic genes. FASN is the enzyme the catalyzes the synthesis of long-chain fatty acid from acetyl-CoA, malonyl-CoA and NADPH. Both proteins have been related to the pathogenesis of NAFLD [121]. Our results showed that *Srebp1c* does not present significant changes after TM treatment in both intervention groups (Figure R6B). *Fasn* was downregulated after TM injection in both LoxP and LFKO mice compared with their correspondent control group (Figure R6C).

In any case, the absence of FGF21 modified the expression pattern caused by TM in any of the genes analyzed. It is worth to mention that the induction of *Dgat1* is in agreement with the TG accumulation observed in the liver of TM mice (Figure R1B). Altogether, these data indicated that *de novo* lipogenesis was repressed under TM treatment, and lipid synthesis was active.


A
Dgat1
Hepatic mRNA relative levels
4
3
2
1
0
####
$$$$
Loxp
FKO
Vehicle
Tunicamycin
B
Srebp1c
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Tunicamycin

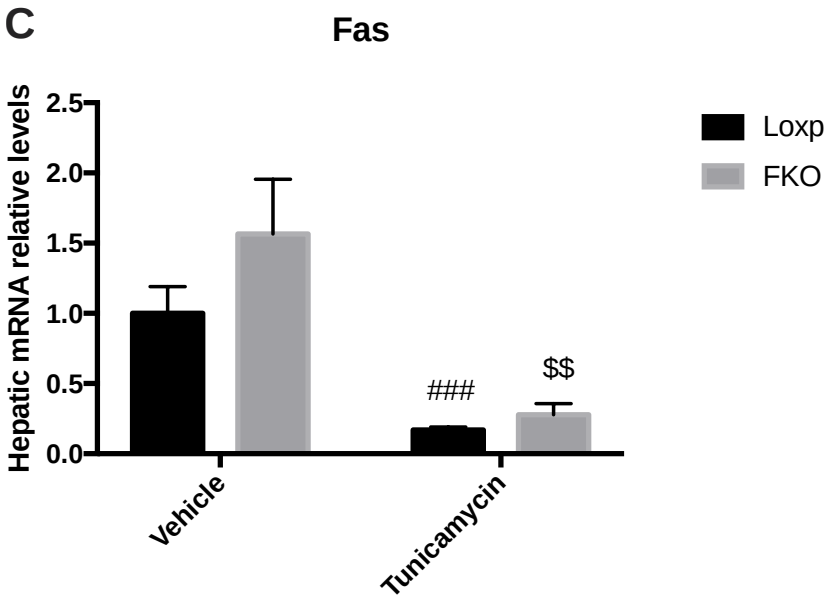


Figure R6. mRNA expression analysis of genes involved in lipid metabolism in liver of LoxP and LFKO mice after 24h of TM or vehicle administration. Gene expression of diacylglycerol O-acyltransferase 1 (*Dgat1*) (A), sterol regulatory element binding protein (*Srebp-1c*) (B), fatty acid synthase (*Fas*) (C). The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs LoxP Vehicle. $p<0.05; $$p<0.01; $$$p<0.001 vs LFKO Vehicle.

The mRNA relative levels of *Fsp27b*, a gene involved in lipid droplet formation in the liver, and *Chrebp*, involved in *de novo* lipogenesis, were measured in the liver of LFKO and LoxP mice. Results showed no changes in the expression of these genes after TM treatment. These indicate that the induction of *Fsp27* or Chrebp does not mediate TM effects over TG storage and that FGF21 is not implied in these responses (Figure R7A, 7B).

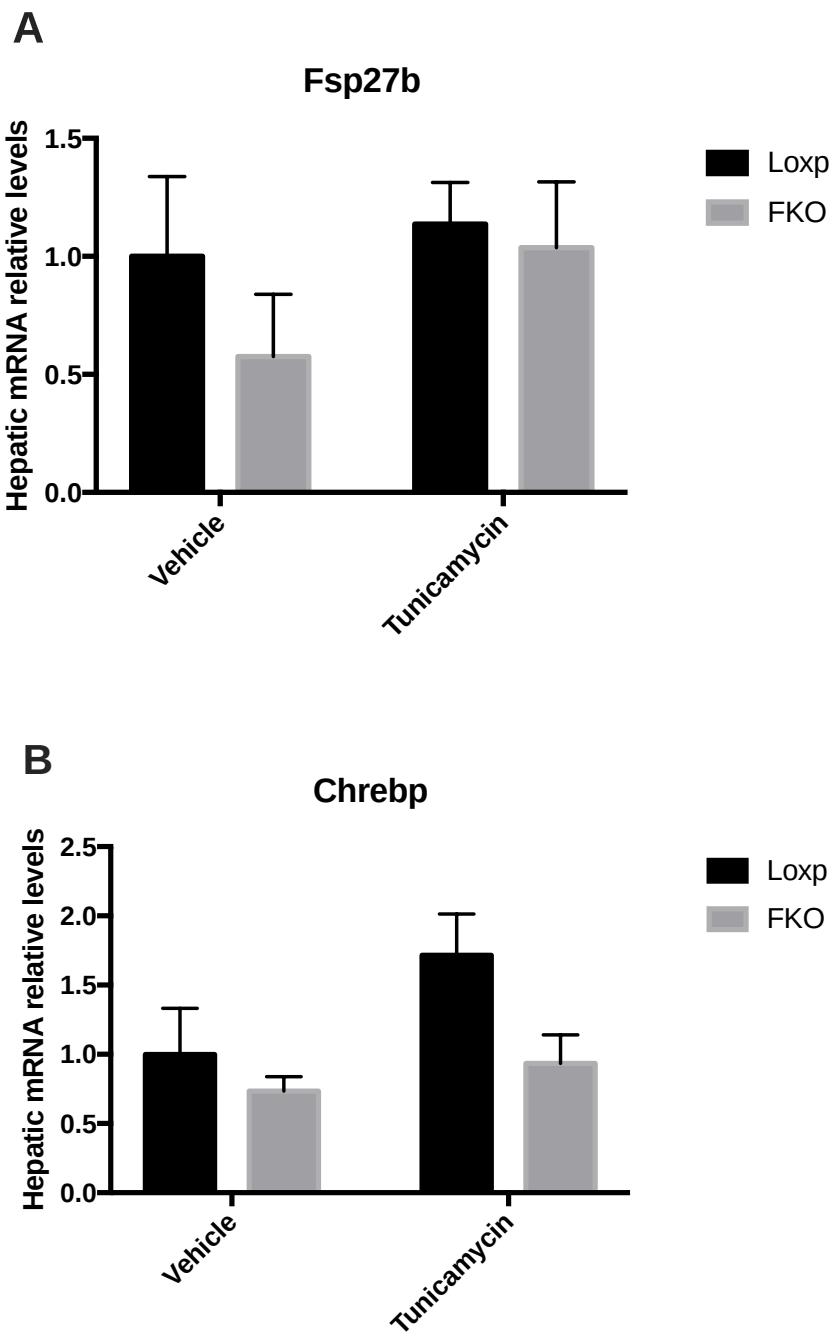


Figure R7. mRNA expression analysis of genes involved in lipid metabolism in liver of LoxP and LFKO mice after 24h of TM or vehicle administration. Gene expression of *Fsp27b* (A) and *Chrebp* (B).

Tunicamycin induced gluconeogenic genes and does not alter ketogenesis after 24h treatment

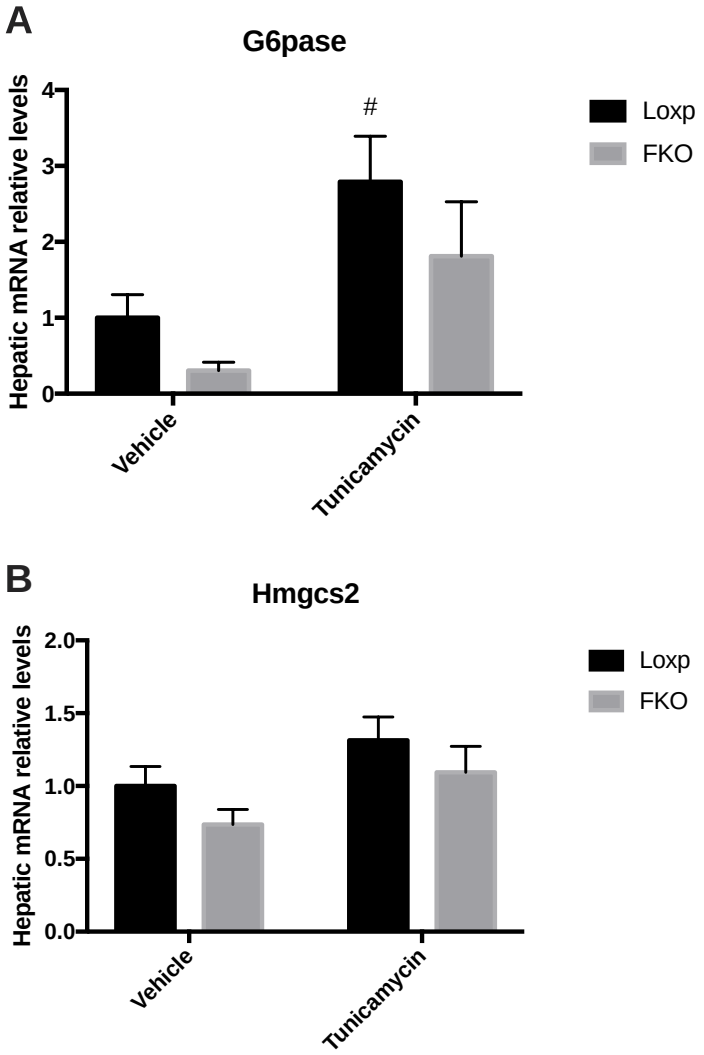
It has been reported that ER stress mediates changes in hepatic gluconeogenesis [139,145], by inducing ATF6 expression which inhibits gluconeogenesis and lipogenesis by binding to CREBH-regulated transcription factor 2 (TORC2) and *Xbp1c-s* that can directly or indirectly activate lipogenesis program while inhibiting gluconeogenesis [139].

Some authors described that TM could inhibit gluconeogenesis within a short-time period [139,145]. TM has been reported to induce glucose metabolism disorders [139], we analyzed the expression of genes involved in gluconeogenesis and ketogenesis in the liver of mice treated with vehicle or TM. Our results demonstrate that TM induces G6Pase in both LoxP and LFKO mice (Figure R8A). The absence of Fgf21 does not affect G6Pase expression in the liver after TM administration.

On the other hand, as changes in the gluconeogenic pathway may affect the response in ketone body production, we analyzed the expression of the *Hmgcs2*, a key enzyme required for ketone body biosynthesis. *Hmgcs2* mRNA levels were not affected by TM injection in LoxP and LFKO mice (Figure R8B). To confirm whether ketone body production was not being affected, B-hydroxybutyrate levels were measured in the liver. Results confirm that the

ketogenesis pathway is not affected by TM injection nor in LoxP nor LFKO mice (Figure R8C).

These results suggest that glucose production is induced by the gluconeogenesis pathway, while ketogenesis is not being affected after 24h of TM treatment. Neither of these responses are mediated by hepatic FGF21.



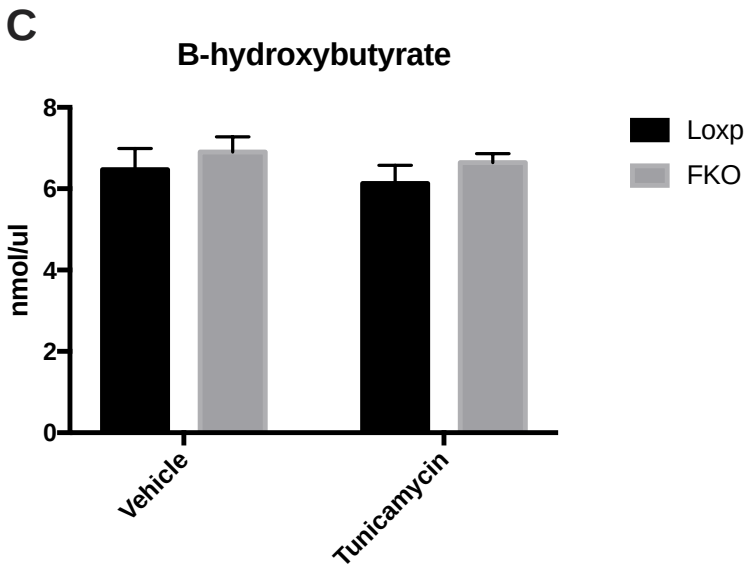


Figure R8. mRNA expression analysis of genes involved in gluconeogenesis and ketogenesis; and B-hydroxybutyrate levels in the liver of LoxP and LFKO mice after 24h of TM or vehicle administration. Gene expression of glucose 6-phosphate (*G6Pase*) (A); 3-hydroxy-3-methylglutaryl-CoA synthase 2 (*Hmgcs2*) (B); and B-hydroxybutyrate levels in the liver (C). The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs LoxP Vehicle.

Chapter 2

Effects of protein restriction mediated by FGF21 to counteract the metabolic outcomes of a high-fat diet

FGF21 has been identified as a key mediator in the metabolic response to an amino acid or protein intake deficiency [13,14,157]. Recent studies published in our laboratory described that under amino acid-deficient diets or low protein diets, there is a reduction of *de novo* lipogenesis in the liver by the downregulation of *Fasn*, and finally, increased expression of the thermogenic genes in BAT and WAT [67,158].

On the other side, obesity is a state in which circulating levels of FGF21 are elevated [128,129]. However, in this situation, endogenous FGF21 levels appear to be ineffective, whereas high pharmacological doses induce its effects, promoting weight loss, improving glucose tolerance, and lowering serum free fatty acids. The role of YIPF6 should be considered during the obesity state, as YIPF6 protein can join to FGF21 and prevent its secretion, leading to a reduction of FGF21 levels in serum [98].

The question is, what will be the impact of a protein reduction within an HFD? We hypothesized that in mice fed with an HFD-LP, there would be an induction of hepatic FGF21 levels and may be an improvement of the FGF21 signaling in its target tissues that could counteract at least in part the metabolic disruptions caused by HFD-induced obesity.

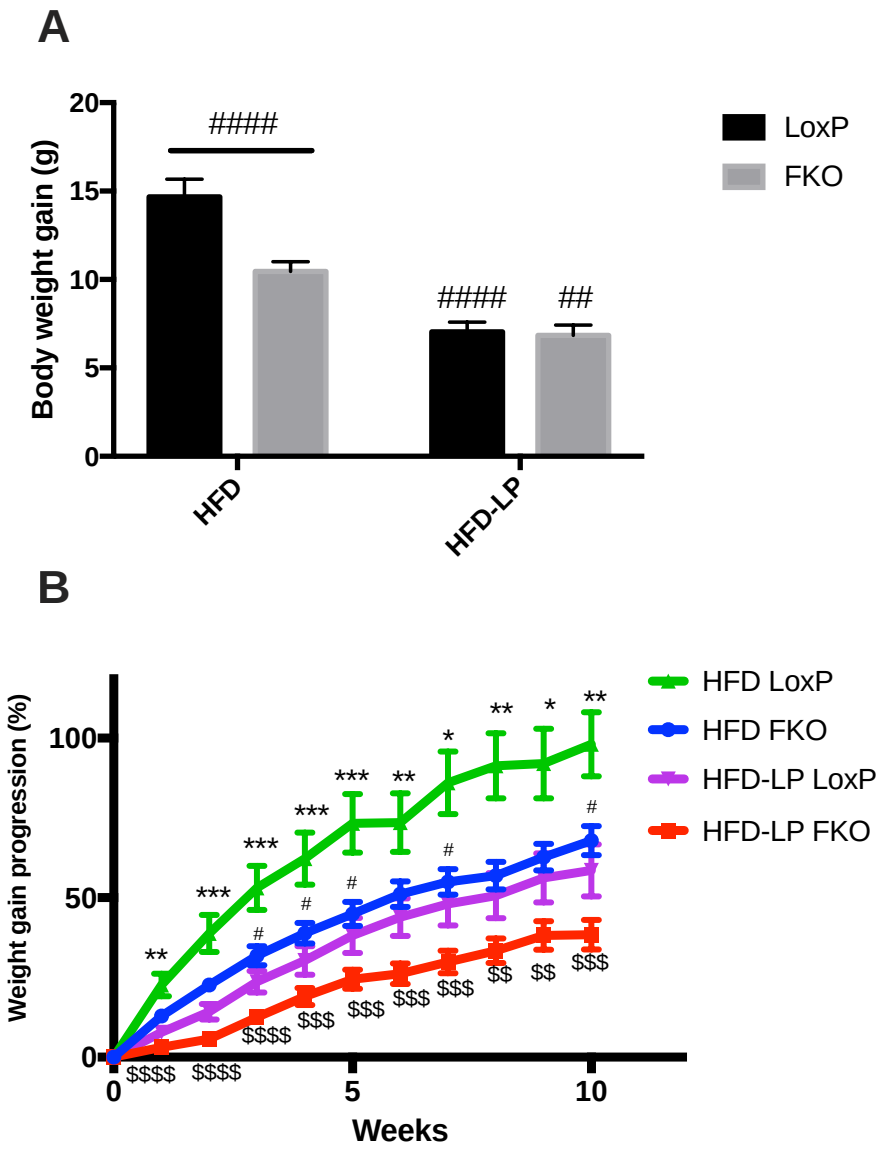
To address the impact of protein restriction within an HFD and the role of FGF21 in this impact, we evaluate the body weight gain, the calorie intake, and the glucose tolerance/insulin sensitivity during the 10-week intervention in mice fed with an HFD or a HFD-LP. Then we analyze the FGF21 expression and signaling and the metabolic profile of these animals in both LoxP and LFKO mice.

Protein restriction requires FGF21 to prevent body weight gain induced by a HFD

Bodyweight progression was evaluated twice a week for the ten weeks of dietary intervention. Bodyweight gain after the nutritional intervention showed that LoxP mice fed with an HFD gain more weight than the LFKO mice in the same diet. Protein restriction intervention prevented body weight gain in both LoxP and LFKO mice, indicating a protective role of this intervention counteracting weight gain caused by a high fat intake (Figure R9A, 9B).

Food intake was measured twice per week during the 10-week intervention. Calorie intake was analyzed, and results showed that calorie intake was similar in LoxP and LFKO mice during an HFD intervention (Figure R9C). Thus, indicating that these mice had a similar calorie intake, LoxP mice showed a marked gain weight compared to the LFKO mice. On the other hand, LoxP mice fed with an HFD-LP had a significantly higher calorie intake during the intervention, indicating that they ate more and presented the same body weight gain than the LFKO mice with lower calorie intake.

These results suggest that protein restriction within an HFD counteract weight gain in an FGF21 dependent manner.



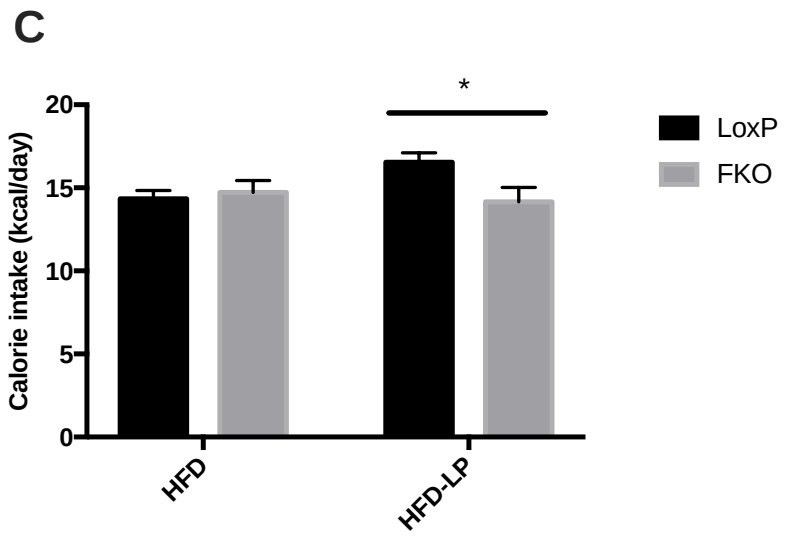
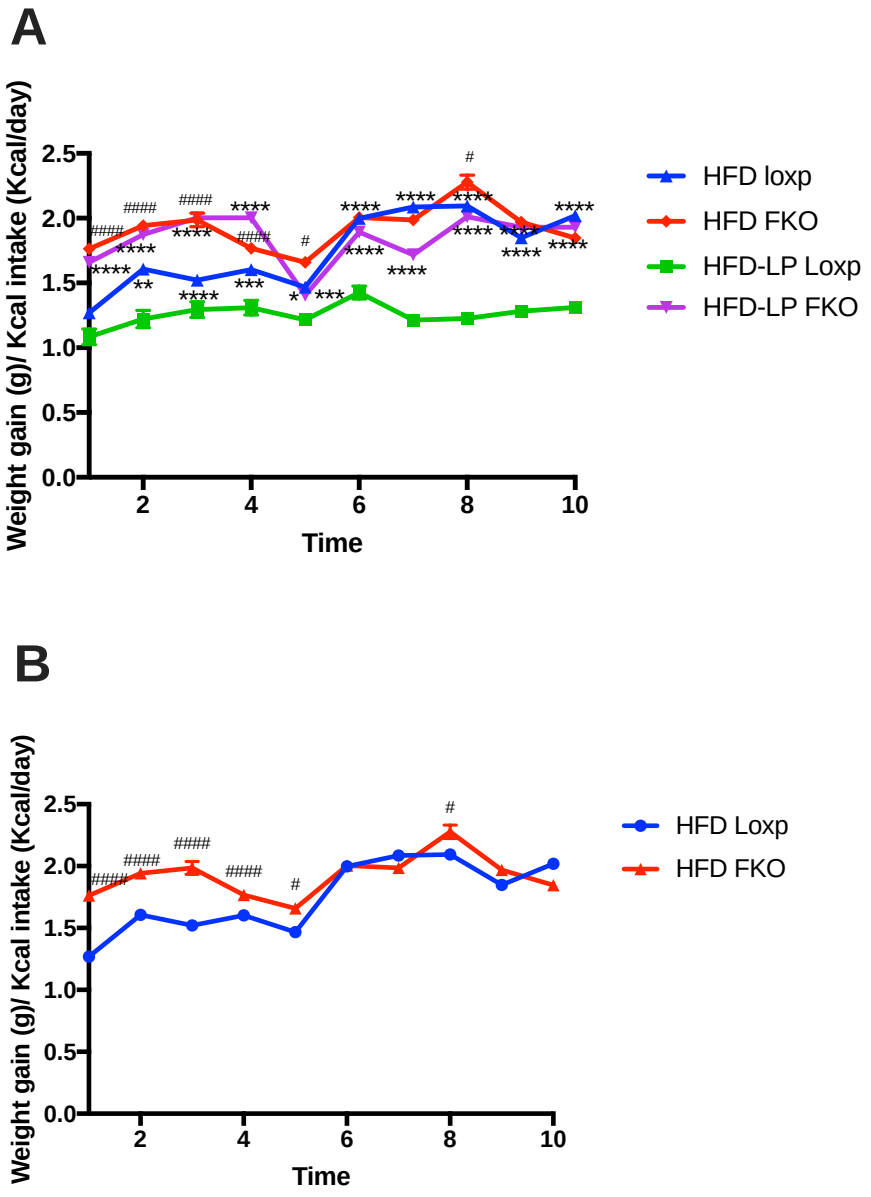


Figure R9. Body weight gain (g) of LoxP and LFKO mice with a HFD or a HFD-LP intervention (A). Body weight gain progression per week (%) during 10-week intervention (B). Average calorie intake per day (C). Food intake and weight were recorded twice per week. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs HFD LoxP. *p<0.05; **p<0.01; ***p<0.001 vs HFD-LP LoxP.

Mice in each intervention group presented a different average in food intake, thus, to normalize body gain with the number of calories ingested we analyzed the relation between body weight gain in grams and calorie intake per week in each intervention group during the 10-week nutritional intervention in LoxP and LFKO mice fed with an HFD or an HFD-LP (Figure R10A).

Figure R10B shows a similar bodyweight progression in both genotypes under an HFD. These results suggest that FGF21 during an HFD intervention does not affect weight gain in mice. On the other hand, under an HFD-LP diet, LFKO mice put on more weight-related to calorie intake than the LoxP group (Figure R10C), thus

indicating that protein restriction, mediated by FGF21, ameliorates the weight gain induced by high fat intake.



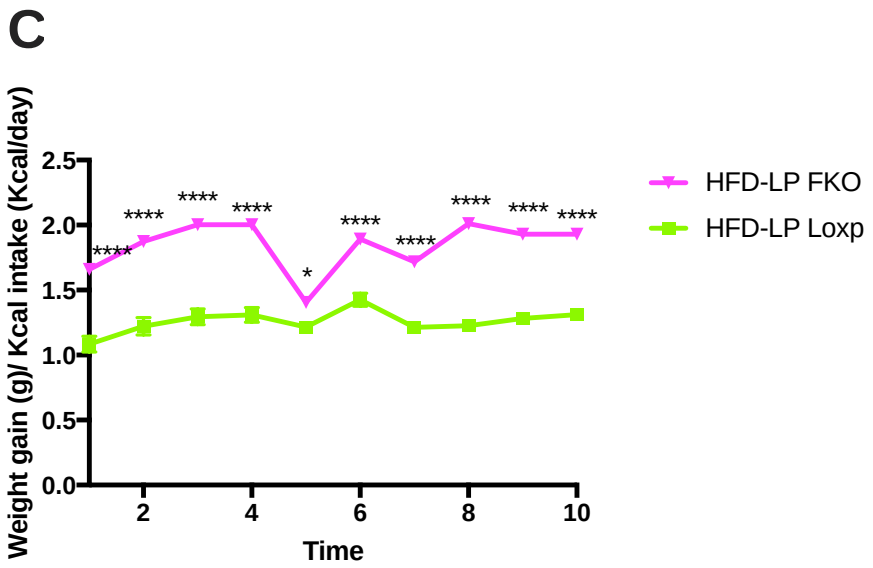


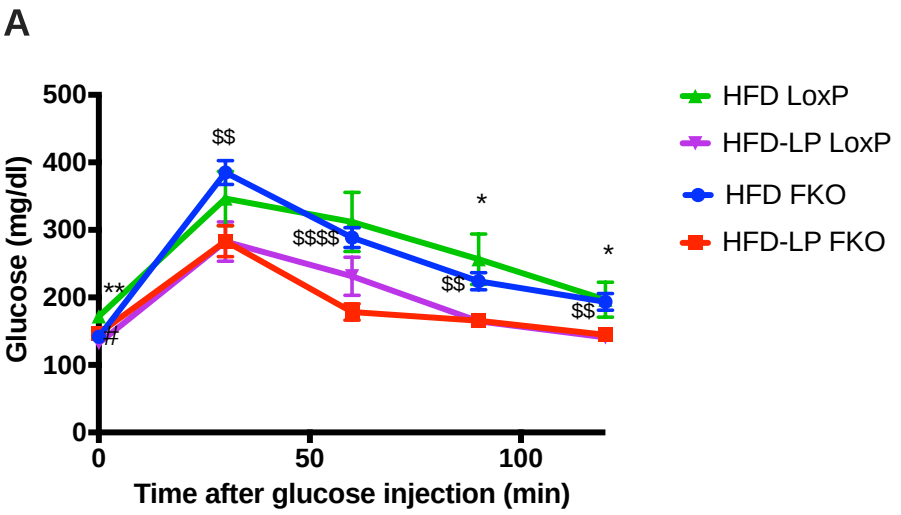
Figure R10. Body weight progression related to calorie intake during a 10-week intervention with an HFD or HFD-LP in LoxP and LFKO mice. Bodyweight gain progression related to calorie intake (kcal) in LoxP and LFKO mice (C). Body weight gain progression related to calorie intake (kcal) of LoxP and LFKO mice fed with an HFD (D) or fed with an HFD-LP (E). Food intake and weight were recorded twice per week. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs HFD LoxP. *p<0.05; **p<0.01; ***p<0.001 vs HFD-LP LoxP.

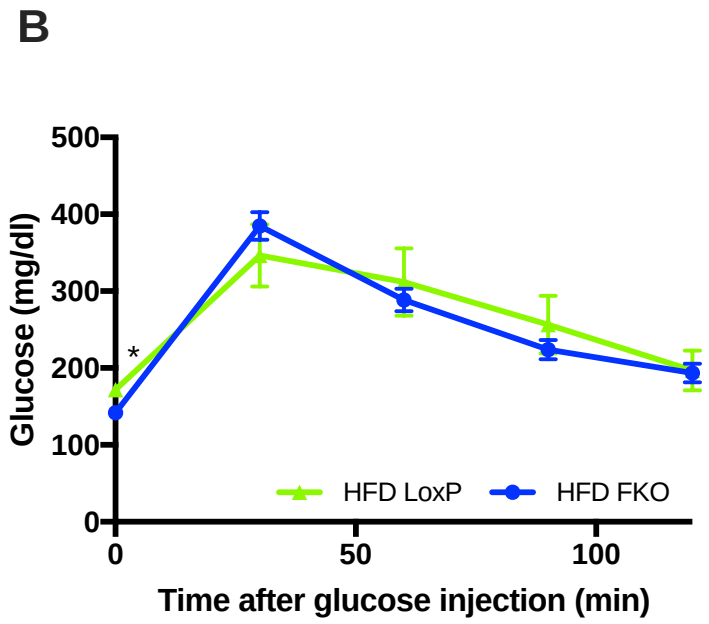
Dietary protein restriction within a HFD improved glucose tolerance in an FGF21-independent way

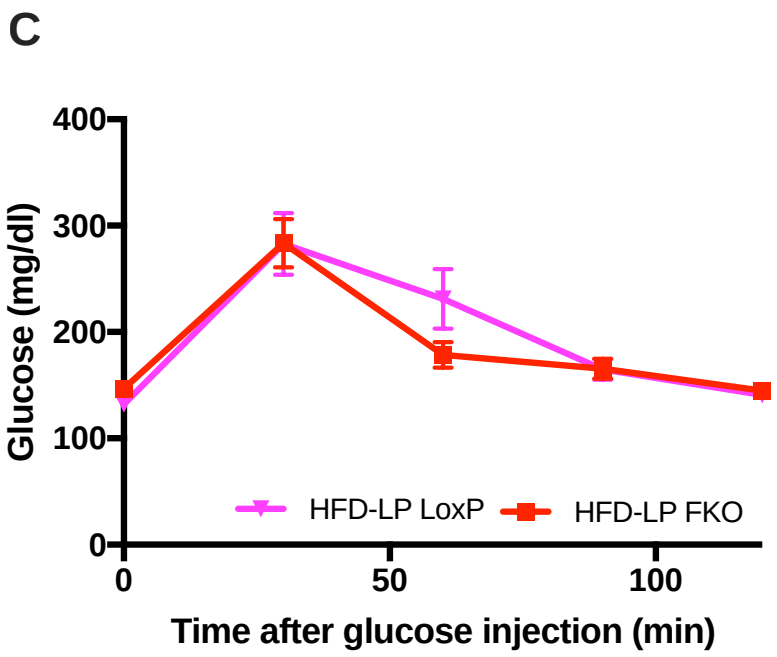
During obesity induced by an HFD, ectopic lipids trigger oxidative stress, inflammation, and cell-signaling events that disrupt insulin action and lead to type 2 diabetes [159]. Insulin insensitivity is apparent within days of high-fat feeding when adiposity, and changes in markers of inflammation are relatively minor [160]. To assess glucose tolerance and insulin sensitivity, a glucose tolerance

test (GTT) at 8-week, and an insulin tolerance test (ITT) at 9-week of the nutritional intervention was carried out.

The GTT curve displayed a better response to glucose in both LoxP and LFKO fed with an HFD-LP compared with their respective controls (Figure R11A). However, no differences between genotypes when fed with an HFD or HFD-LP were detected (Figure R11B, 11C), this indicates that the improvement of the glucose tolerance by protein restriction is not due to the presence of FGF21. These results are confirmed by the area under the curve (AUC), which showed that protein restriction improves glucose levels and counteract glucose intolerance caused by an HFD, but not mediated by FGF21 (Figure R11D).




B
500
400
300
200
100
0
Glucose (mg/dl)
*
HFD LoxP
HFD FKO
0
50
100
Time after glucose injection (min)


C
400
300
200
100
0
Glucose (mg/dl)
HFD-LP LoxP
HFD-LP FKO
0
50
100
Time after glucose injection (min)

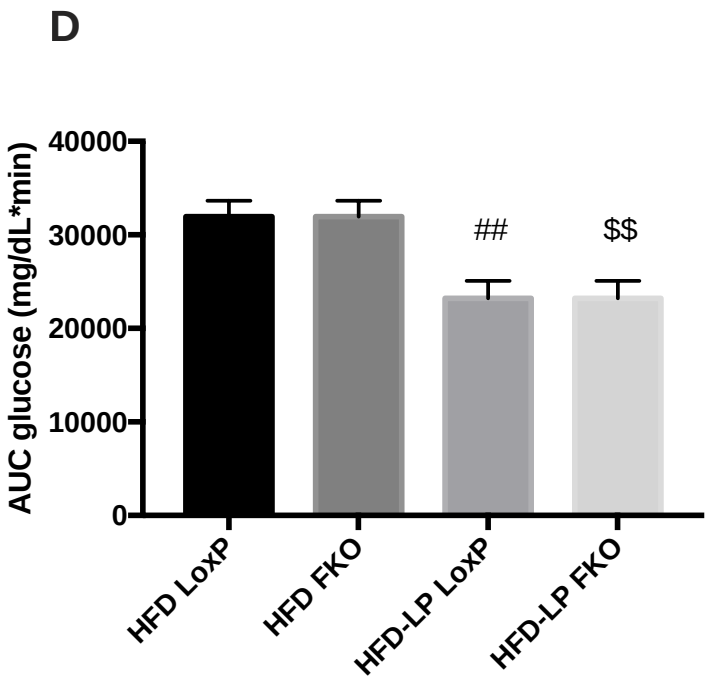


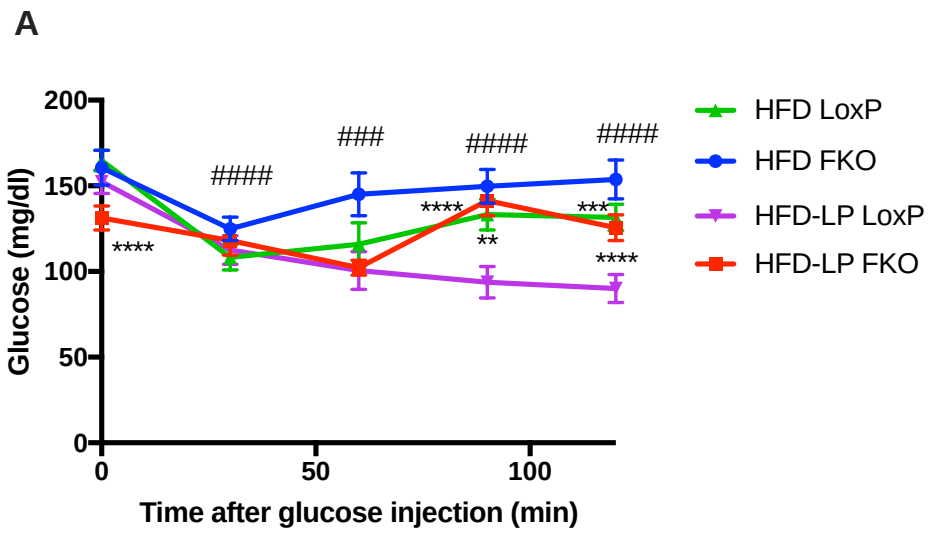
Figure R11. A low protein intake within a HFD improved glucose tolerance not mediated by hepatic Fgf21. GTT curve showing plasma glucose levels after i.p. administration of glucose (1.5 g/kg/ b.w.) in HFD or HFD-LP LoxP or LFKO mice after 8 weeks of nutritional intervention (A). GTT of LoxP and LFKO mice fed with a HFD (B) or a HFD-LP (C); Area under the curve (AUC) of LoxP and LFKO mice fed with a HFD or a HFD-LP (D). The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs HFD LoxP. $p<0.05; $$p<0.01; $$$p<0.001 vs HFD LFKO. *p<0.05; **p<0.01; ***p<0.001 vs HFD-LP LoxP.

Protein restriction required FGF21 to improve insulin sensitivity and to counteract part of the effects caused by an HFD-induced obesity

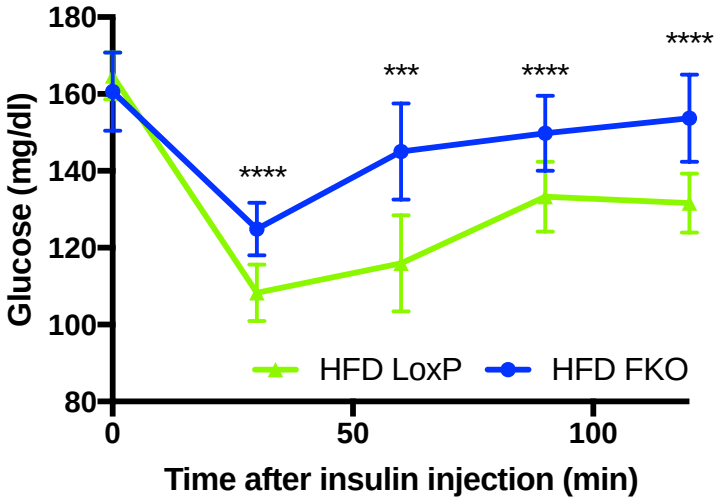
An ITT was carried out at the 9-week dietary intervention in LoxP and LFKO mice fed with an HFD or an HFD-LP. Our results showed that protein restriction improved the insulin response in both LoxP

and LFKO mice compared to their respective controls (Figure R12A). This improvement is more considerable in LoxP mice than in LFKO, thus indicating a role of FGF21 on this response (Figure R12B).

Similarly, LFKO mice fed with an HFD exhibited a worse insulin response (Figure R12C) that LoxP mice fed an HFD. The glucose levels of LFKO animals were higher in each time point after the insulin injection in comparison to the HFD LoxP mice. Altogether these data suggested that protein restriction within HFD improved insulin sensitivity in HFD-fed mice and that this positive effect depends at least in part of the presence of FGF21.



B


180
160
140
120
100
80
Glucose (mg/dl)
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***
****
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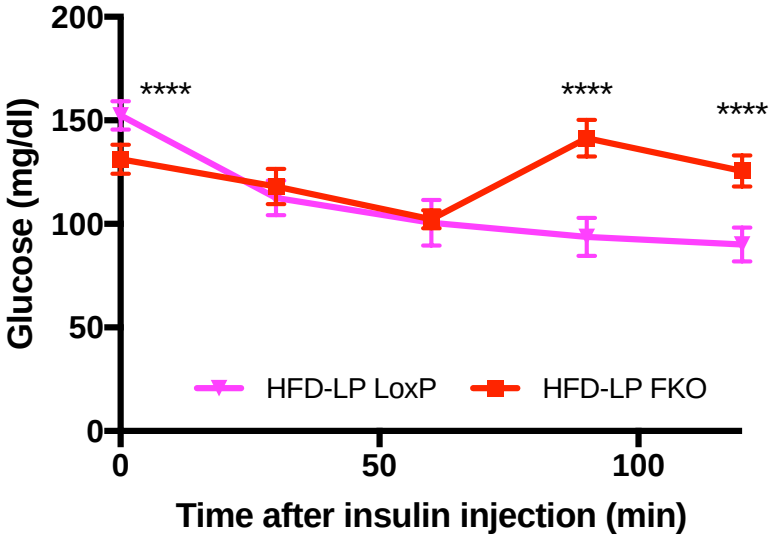
HFD LoxP
HFD FKO
0
50
100
Time after insulin injection (min)

C


200
150
100
50
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Glucose (mg/dl)
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HFD-LP LoxP
HFD-LP FKO
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50
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Time after insulin injection (min)

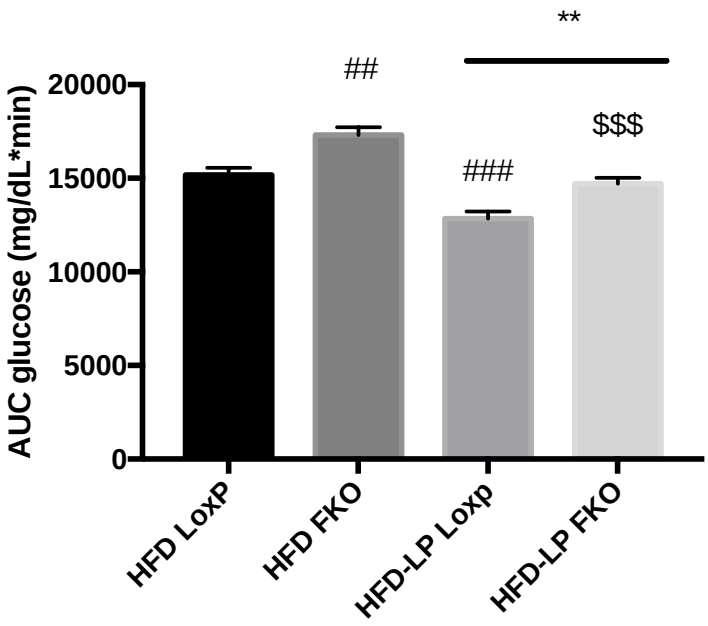


Figure R12. Protein restriction improves insulin sensitivity and counteract insulin resistance caused by a HFD in an FGF21 mediated pathway. ITT curve showing plasma glucose levels after i.p. administration of insulin (0.5 UI/kg b.w.) in HFD and HFD-LP mice after 10 weeks of nutritional intervention (A, B, C). Area under the curve (AUC) of ITT in LoxP and LFKO mice fed with a HFD or a HFD-LP (D). The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs HFD LoxP. $p<0.05; $$p<0.01; $$$p<0.001 vs HFD LFKO. *p<0.05; **p<0.01; ***p<0.001 vs HFD-LP LoxP.

Protein restriction within a high-fat diet increased FGF21 mRNA expression in liver and protein levels in serum

To address the effects of protein restriction within an HFD, we analyzed *Fgf21* mRNA relative expression in the liver of HFD-LP and HFD groups. The results showed that *Fgf21* expression is markedly induced under an HFD-LP intervention (Figure R13A), compared to mice fed with regular protein intake within an HFD.

Likewise, FGF21 serum protein levels were considerably increased in the HFD-LP mice compared to the HFD group (Figure R13B).

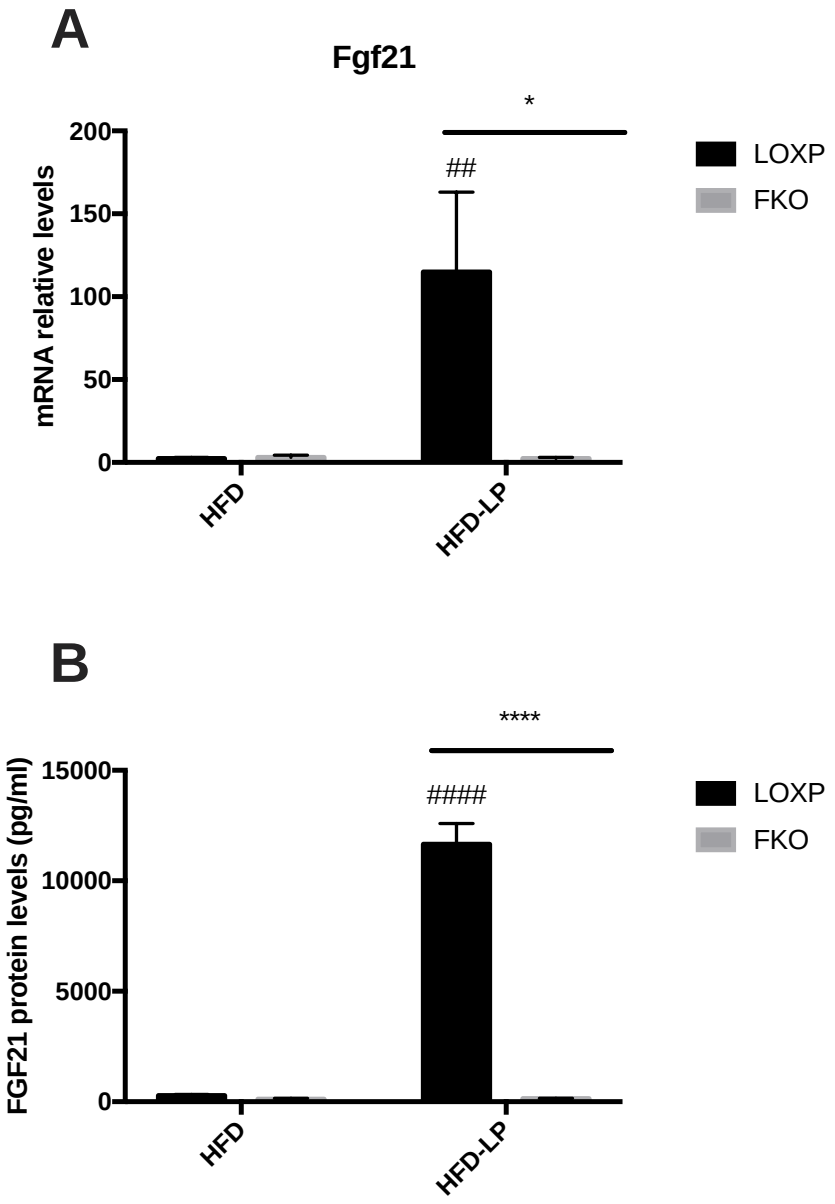


Figure R 13. Fgf21 relative mRNA levels in the liver (A). FGF21 protein levels in serum of HFD-LP and HFD groups of LoxP and LFKO mice after 10 weeks of nutritional intervention

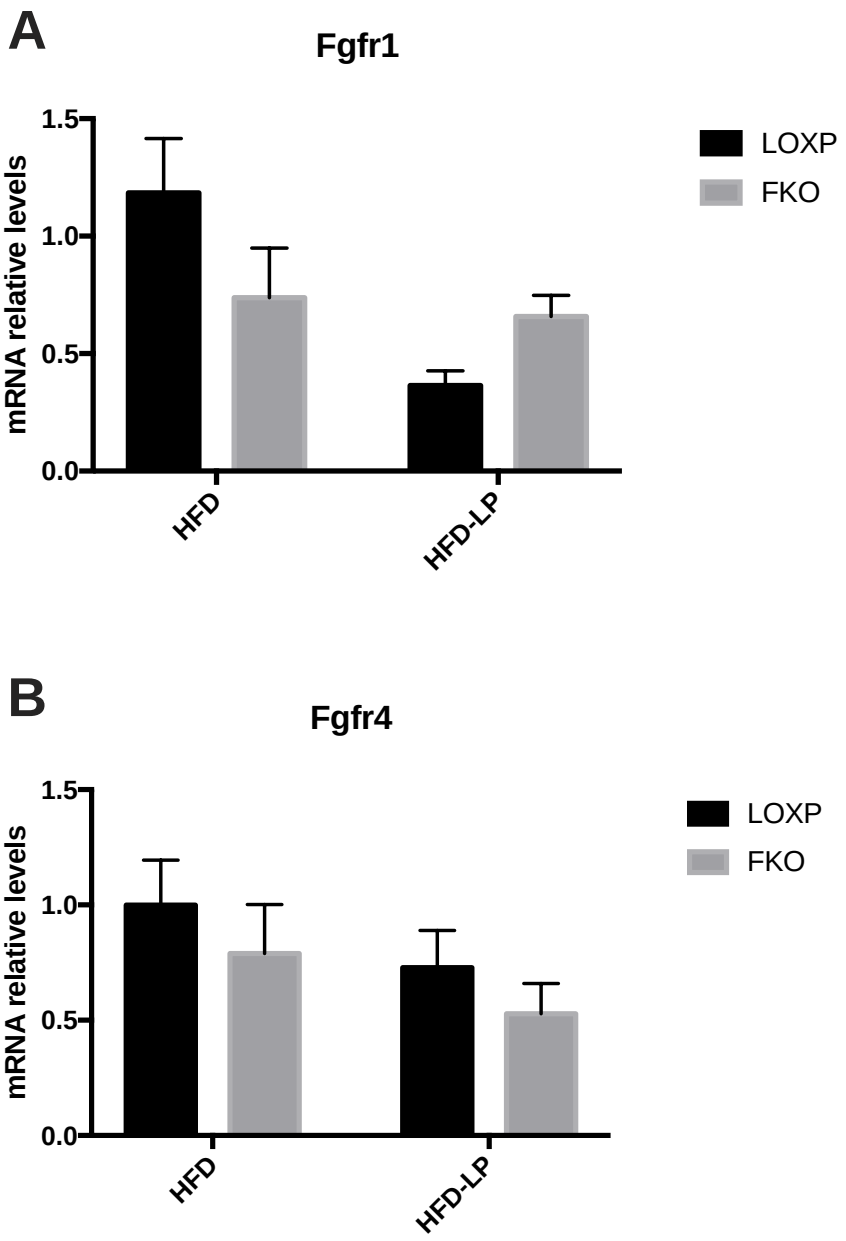
(B). Bars represent the fold induction in the mRNA levels versus the HFD mice that are considered the control group, which produces an arbitrary value of 1. The graph represents the mean +/- SEM. *p<0.05; **p<0.01; **p<0.001 vs HFD LoxP.

FGF21 signaling in the liver is not affected by protein restriction during a HFD-induced obesity

The levels of *FgfR1, FgfR4, and Klb* in the liver were measured to investigate if protein restriction can improve FGF21 signaling during HFD-induced obesity and if it is signaling in the liver can ameliorate the metabolic disturbances caused by an HFD.

The results indicated that LoxP mice fed an HFD showed higher *Fgfr1* mRNA levels than LoxP mice fed with HFD-LP. Regarding LFKO mice, no significant changes in *Fgfr1* levels when fed with an HFD or a protein-restricted diet were observed (Figure R14A). However, under HFD-LP, LFKO mice exhibited higher mRNA levels of Fgfr1 than the LoxP mice (Figure 11A). No differences in *Fgfr4* were detected in the liver of LoxP and LFKO mice in any dietary intervention (Figure R14B)

Finally, *Klb* mRNA levels showed a tendency to rise during a protein-restricted diet, and this induction is even higher in LFKO mice compared to the LoxP mice fed HFD-LP (Figure R14C).


A
Fgfr1
mRNA relative levels
1.5
1.0
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LOXP
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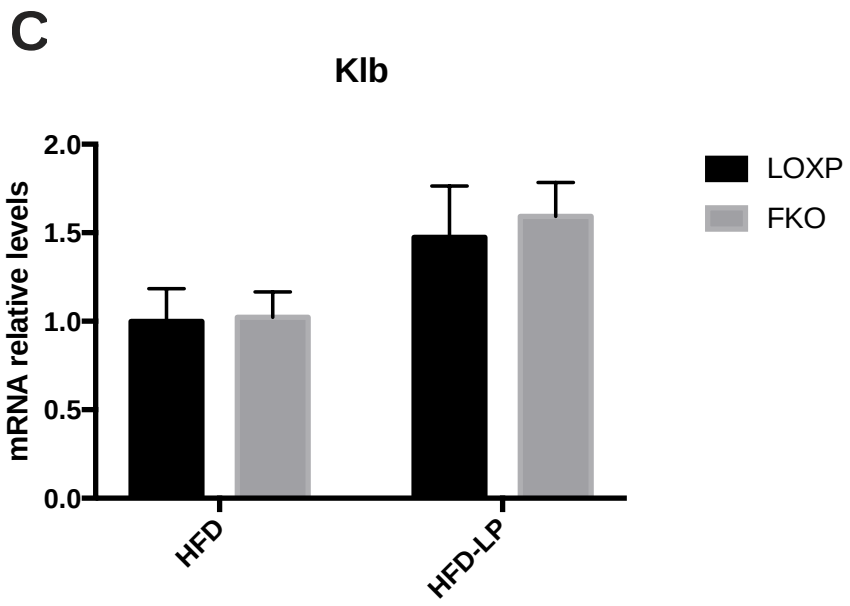


Figure R14. Fgf21 receptors in the liver. *Fgfr1* (A)*, Fgfr4* (B)*,* and *Klb* (C) mRNA levels in the livers of mice fed with a HFD or a HFD-LP. Bars represent the fold induction in the mRNA levels versus the HFD mice that are considered the control group, which produces an arbitrary value of 1. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs HFD LoxP. *p<0.05; **p<0.01; ***p<0.001 vs HFD-LP LoxP.

Protein restriction requires hepatic FGF21 to prevent lipid storage in the liver during a HFD-induced obesity

After 10-weeks of nutritional intervention, mice were sacrificed, and the liver was extracted and weighted. Our results showed that protein restriction within an HFD caused a significant reduction in LoxP mice's liver weight compared to the HFD LoxP mice (Figure R15A). This reduction is dependent on hepatic FGF21 as these effects were not observed in LFKO mice with an HFD-LP intervention (Figure R15B). The put-on weight of the liver in LFKO

mice fed with an HFD-LP is similar to that observed in mice fed an HFD.

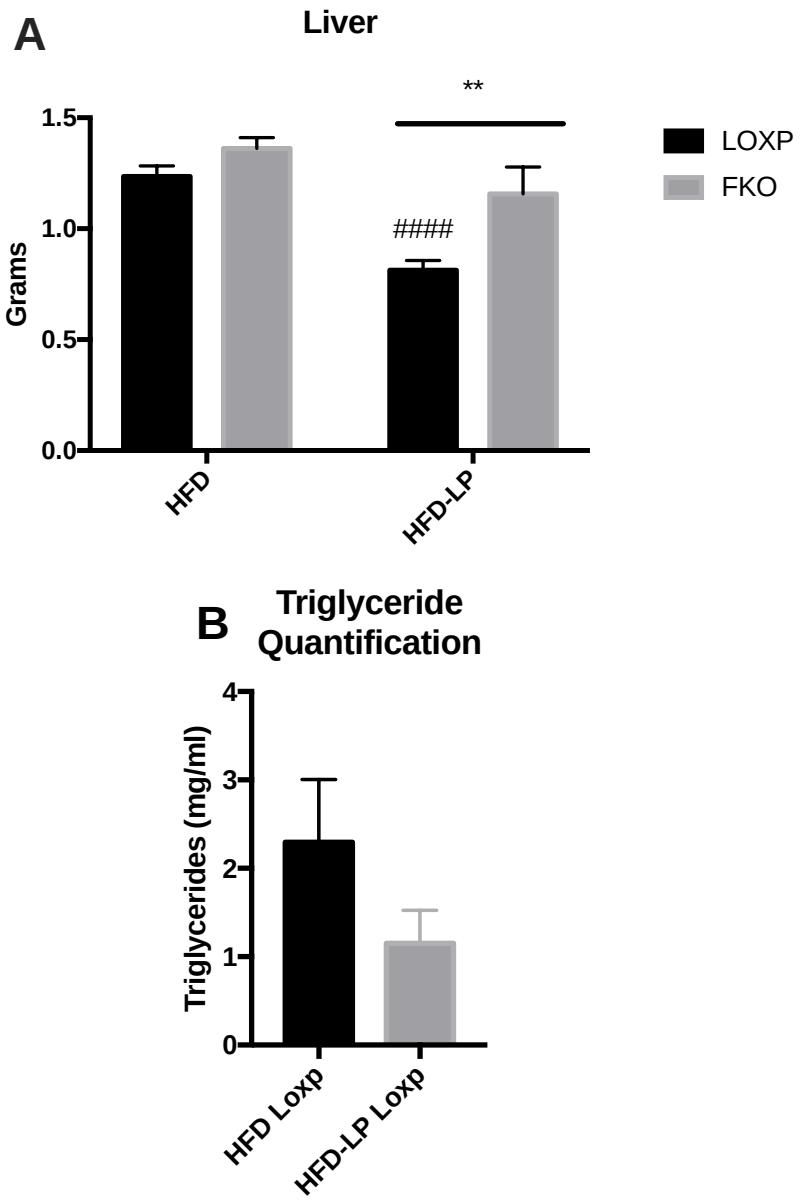
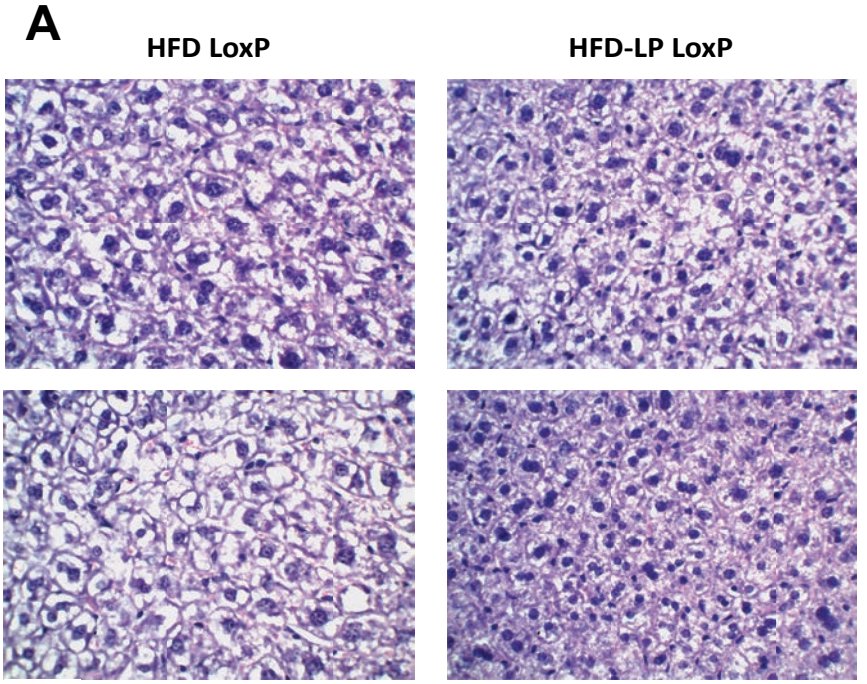


Figure R15. A low protein diet reduces liver weight and lipid storage. Liver weight in LFKO and LoxP mice with a HFD or a HFD-LP intervention(A); Liver triglyceride quantification in LoxP mice fed with a HFD or a HFD-LP intervention (B). The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs HFD Loxp. *p<0.05; **p<0.01; ***p<0.001 vs HFD-LP Loxp.

To analyze if this reduction is due to changes in the hepatic lipid content, a triglyceride quantification analysis in the liver of LoxP and LFKO mice fed with an HFD or an HFD-LP intervention was carried out. Results showed that the HFD-LP group tended to store fewer triglycerides than the HFD LoxP group (Figure R 13B). This data is supported by the representative hematoxylin and eosin (H&E)-stained liver sections, which show lower lipid content in hepatocytes of mice fed with an HFD-LP (Figure R16A, 16B).



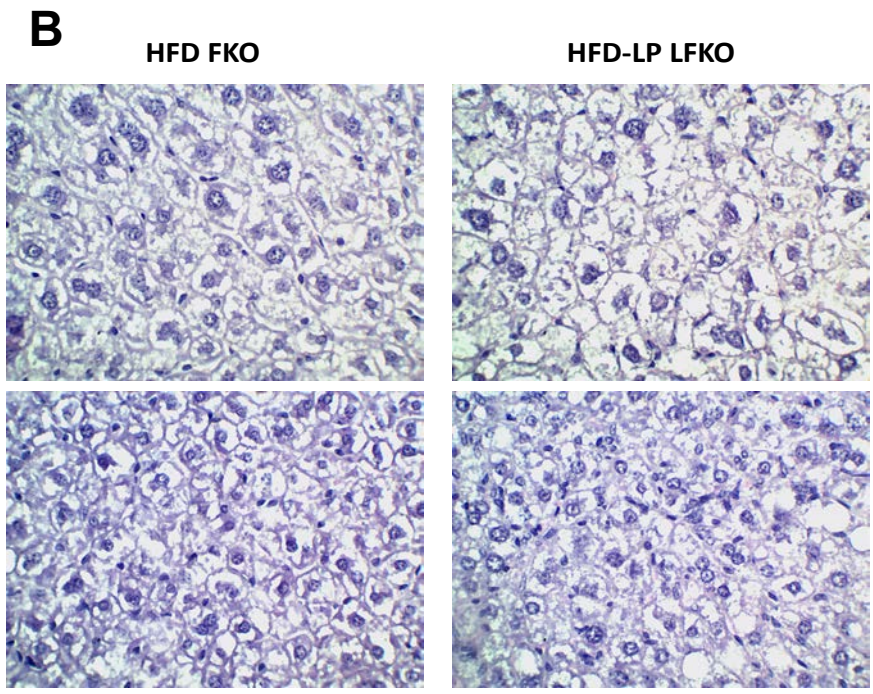


Figure R16. Representative hematoxylin and eosin (H&E)-stained liver sections from LoxP (A) and LFKO mice (B) fed with an HFD and HFD-LP.

A high-fat overload represses *de novo* lipogenesis in the liver, although FGF21 mediates the hepatic downregulation of fatty acid transport and lipid droplet formation.

During hepatic TAG formation, fatty acids are derived from the diet, *de novo* lipogenesis, and adipose tissue via lipolysis. Once in hepatocytes, free fatty acids can further be oxidized in mitochondria via the ß-oxidation pathway, re-esterified to TAG, and stored in lipid droplets or coupled to apolipoproteins and further secreted as very-low-density lipoprotein (VLDL) [161].

Intracellular lipids and their synthesis contribute to the mechanisms and complications of obesity-associated diseases. Despite insulin resistance, insulin and inflammation continue to induce lipogenic genes in the liver. It has been described that in this context, SREBP1c is over-expressed in the liver [162] contributing to the pathogenesis of NAFLD [159].

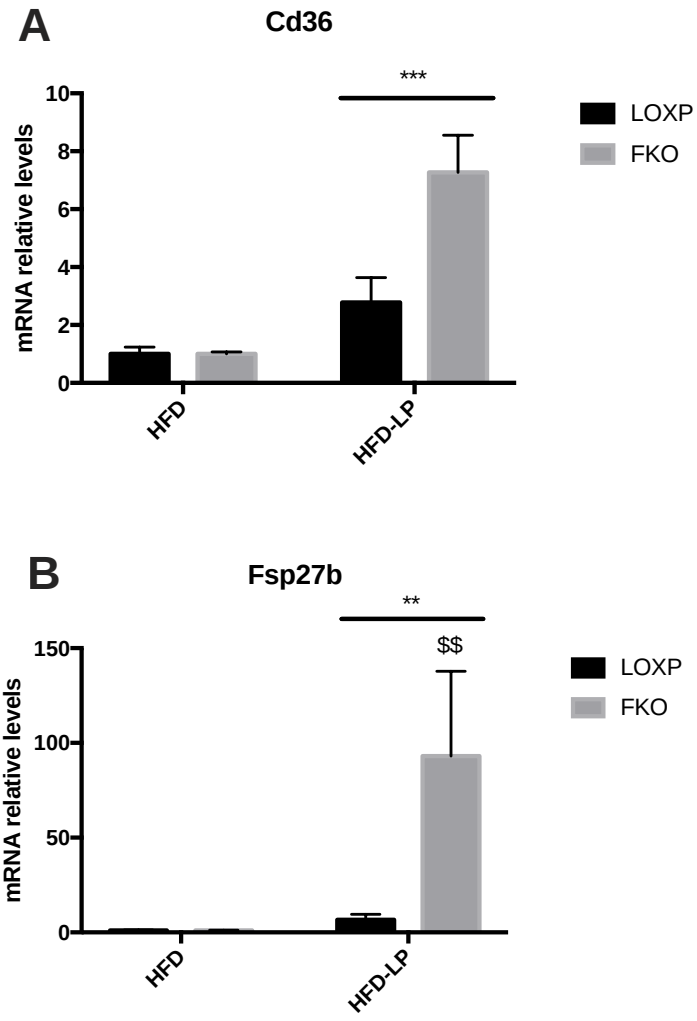
Fat-specific protein 27 (FSP27), also known as CIDEC in humans, belongs to the cell death-inducing DNA fragmentation factor-like effector family of proteins [163]. FSP27 is highly induced during hepatic steatosis, an obesity-related metabolic pathology [164]. It has been described that FSP27 is a lipid droplet associated protein that promotes lipid droplet formation, growth, and triglyceride storage in white adipocytes [163]. White adipose tissue and the liver specifically expressed Fsp27a and Fsp27b transcripts driven by different promoters [165].

To identify if *de novo* lipogenesis is induced in HFD-Lp intervention, we performed a gene expression analysis of genes involved in fatty acid transport (cluster of differentiation *(Cd36*)), fatty acid synthesis (*Srebp1c*), and lipid droplet formation (fat specific protein 27 b (*Fsp27b))* in the liver of LoxP and LFKO mice fed with an HFD or an HFD-LP (Figure R17).

Results showed that protein restriction mediated by FGF21 represses fatty acid transport and lipid droplet formation by repressing the mRNA expression of *Cd36* and *Fsp27b* in LoxP mice

fed with an HFD-LP in comparison to the LFKO mice with the same nutritional intervention (Figure R17A,17B).

This data indicate that protein restriction mediated by FGF21 suppresses fatty acid transport, and lipid droplet formation during a high-fat intake. By contrast, the expression of *Srebp1c* is reduced in both groups fed with an HFD-LP (Figure R17C), thus no being affected by FGF21.



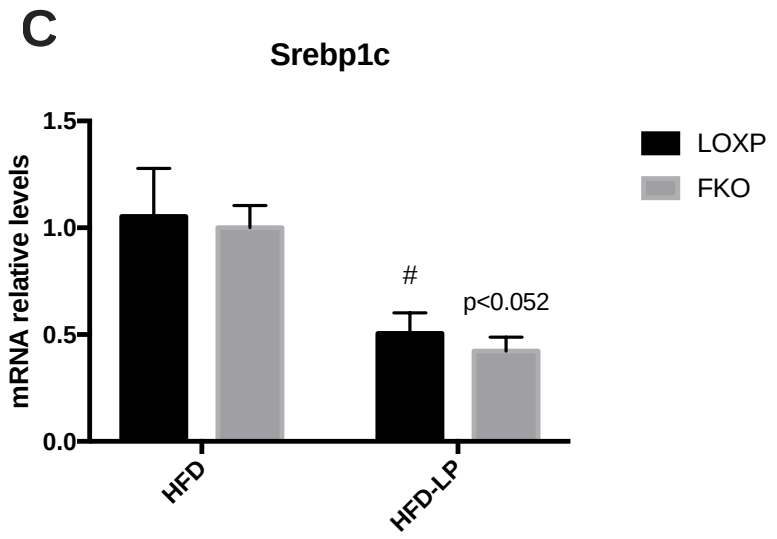


Figure R17. Gene expression analysis in the liver of Loxp and LFKO mice fed with a HFD or a HFD-LP intervention for 10-week of nutritional intervention. mRNA relative levels of cluster of differentiation 36 (Cd36) (A), fat-specific protein 27b (B), sterol regulatory element-binding protein-1c (C). The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs HFD Loxp. $p<0.05; $$p<0.01; $$$p<0.001 vs HFD LFKO. *p<0.05; **p<0.01; ***p<0.001 vs HFD-LP Loxp.

Gluconeogenesis and lipid oxidation are not induced by a HFD or a HFD-LP intervention in the liver

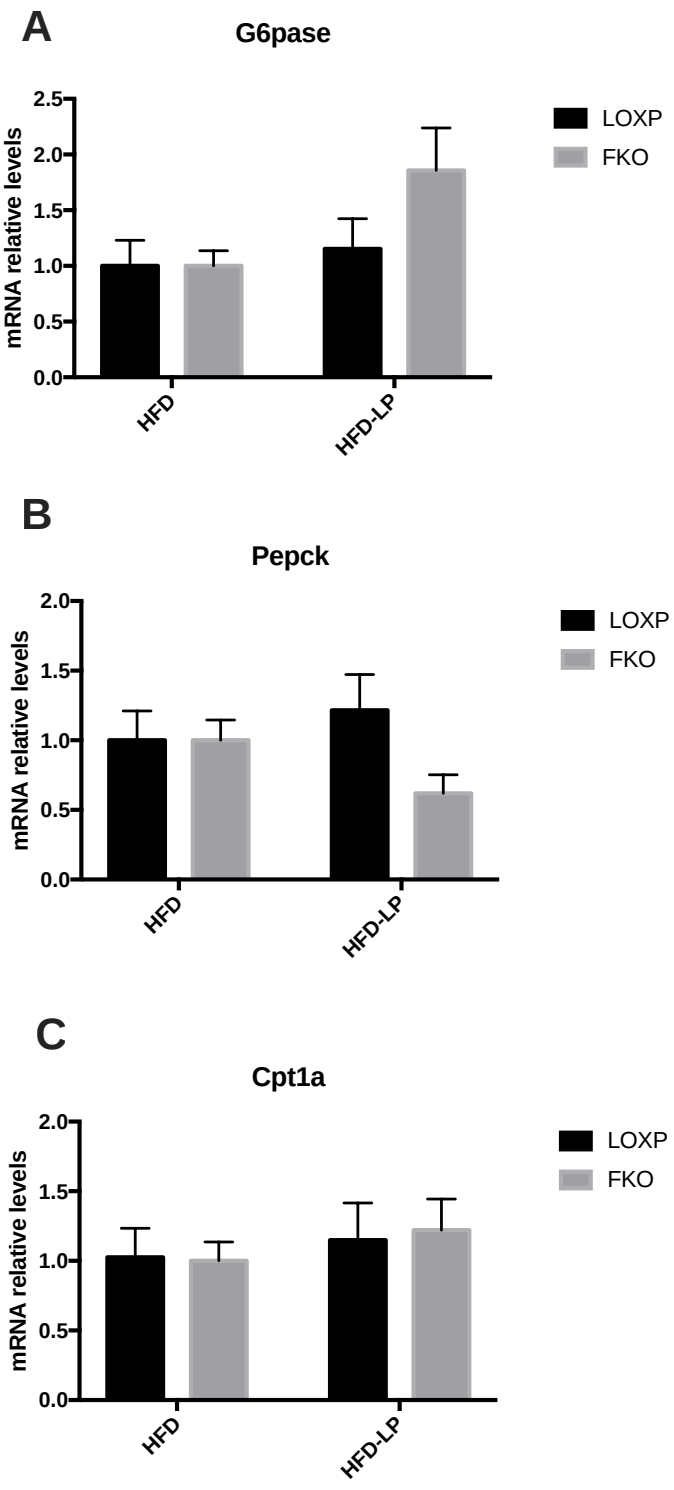
Lipid oxidation is the principal source of energy production in the liver. The dysregulation of the flow of fatty acids from adipose tissue to the liver has been associated with NAFLD's pathogenesis, insulin resistance, and cellular damage [161,166]. The stimulation of lipid oxidation is necessary for the endergonic steps of gluconeogenesis localized in the liver mitochondria, which is upregulated during insulin resistance. Increased activity of the gluconeogenesis pathway has been related to NAFLD. A chronic stimulation of

mitochondria, because of an excessive lipid overload, can produce oxidative stress and thus contributing to the progression of NASH [167].

Therefore, we measured the expression of genes involved in gluconeogenesis and fatty acid oxidation in the liver of LoxP and LFKO mice fed with an HFD or an HFD-LP. The mRNA relative levels of *G6pase* and *Pepck* were not induced by an HFD nor an HFD-LP in LoxP or LFKO mice (Figure R18A and 18B).

Also, carnitine palmitoyltransferase 1A (*Cpt1a*), a key enzyme for fatty acid oxidation, was measured in LoxP and LFKO mice fed with an HFD or an HFD-LP intervention. Results showed that *either of the interventions did not induce Cpt1a* in the liver (Figure R18C).

Altogether, these results showed that gluconeogenesis and fatty acid oxidation are not induced during the metabolic response to protein restriction within HFD-induced obesity.


A
G6pase
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HFD-LP
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FKO
B
Pepck
mRNA relative levels
2.0
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HFD
HFD-LP
LOXP
FKO
C
Cpt1a
mRNA relative levels
2.0
1.5
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HFD
HFD-LP
LOXP
FKO

Figure R18. Gene expression analysis in the liver of Loxp and LFKO mice fed with a HFD or a HFD-LP for 10-week of nutritional intervention. mRNA relative levels of glucose 6-phospatase (*G6pase*) (A), phosphoenolpyruvate carboxykinase (*Pepck*) (B), and carnitine palmitoyl transferase 1A (*Cpt1a*) (C). The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs HFD Loxp. $p<0.05; $$p<0.01; $$$p<0.001 vs HFD LFKO. *p<0.05; **p<0.01; ***p<0.001 vs HFD-LP Loxp.

mTORC1 is not affected by protein restriction in the setting of diet-induced obesity

Then we considered the mechanistic target of rapamycin complex 1 (mTORC1) in this response during protein restriction. mTORC1 is a kinase that mediates key cellular functions and metabolism [168]. mTORC1 is activated by amino acid intake, and disruptions in its signaling have been related to obesity and NAFLD. Also, mTORC1 has been related to the promotion of lipid biosynthesis through SREBPs induction [169].

Protein restriction activates the GCN2/eIF2a/ATF4/5 cascade leading to FGF21 induction. GNC2 also suppresses mTOC1 activation. A low protein diet, mediated by GH/IGF-1 signaling, suppresses mTORC1 activation, and FOXO activation by Akt suppression [170].

Thus, it analyzed the mTORC1 protein activity by measuring the levels and phosphorylation state of its downstream target, the ribosomal protein S6 kinase (S6K) by western blot (Figure R19). The results showed no significant changes in the pS6K/S6K ratio (Figure R19A, 19B) in the liver of LoxP mice in a protein-restricted diet

compared with the HFD intervention group or an HFD-LP. These results suggested that mTORC1c does not play a key role in mediating the effects of protein restriction during HFD-induced obesity in mice.

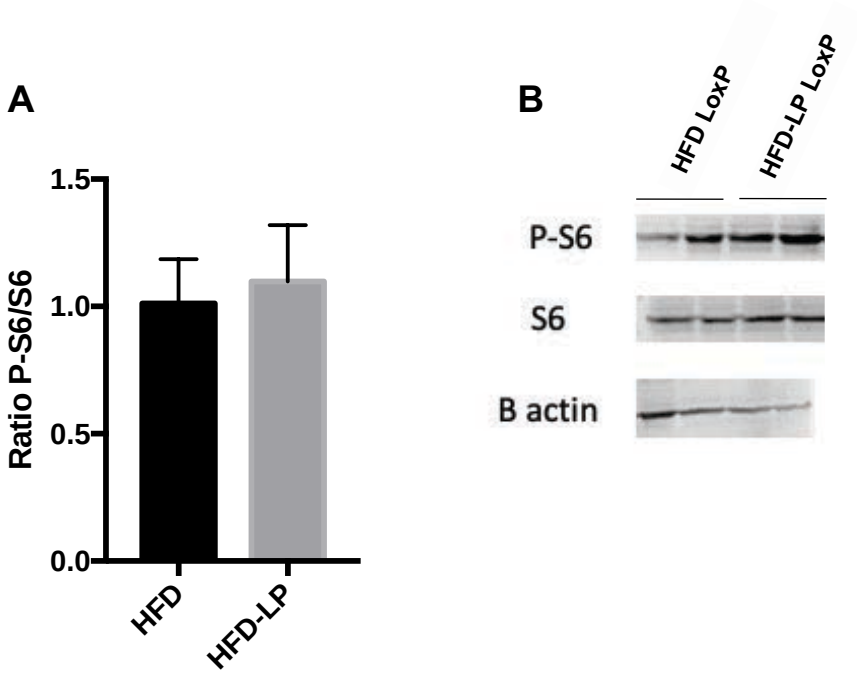


Figure R19. Ratio P-S6/S6 in the liver or LoxP mice fed with a HFD or HFD-LP intervention (A), protein levels were measured with immunoblotting with the mentioned antibodies (B).

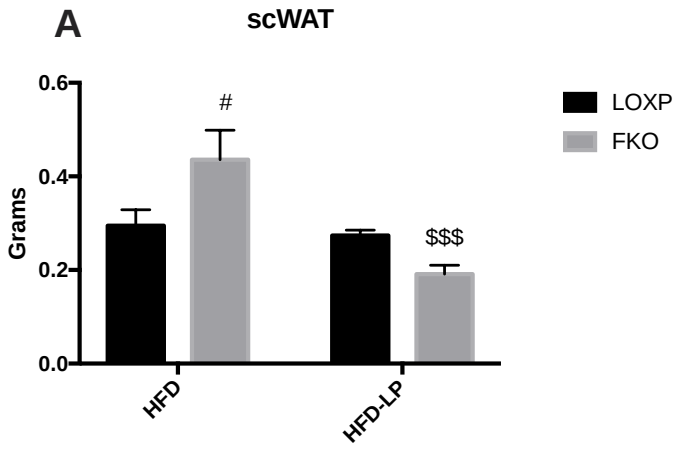
The absence of FGF21 increased fat deposition in WAT under HFD but no within protein restriction

It has been described recently that increased endogenous FGF21 levels during diet-induced obesity may serve as a mechanism to counteract systemic insulin resistance, suggesting that insulin sensitivity improvement was associated with a specific expansion of subcutaneous fat mas [169]. To address if protein restriction could

promote healthy TAG storage during HFD-induced obesity, the subcutaneous WAT (scWAT), epididymal WAT (eWAT), gastrocnemius (gastro), and heart of LoxP and LFKO mice fed with an HFD or an HFD-LP were extracted and immediately weighted after the 10-week nutritional intervention (Figure R20).

LFKO mice fed an HFD exhibited a markedly higher weight in scWAT and eWAT compared with the other experimental groups (Figure R20A and R20B). By contrast, LFKO mice fed with an HFD-LP displayed a lighter scWAT and eWAT in comparison to the LFKO HFD group. Regarding LoxP mice, both nutritional interventions have the same effect in WAT depots analyzed.

Gastrocnemius and heart were immediately weighted after sacrifice. Results showed that protein restriction prevented gastrocnemius weight gain in the LoxP mice compared with the LoxP HFD group (Figure R20C). LFKO mice fed with an HFD-LP maintain the same weight as the LFKO HFD group. The heart weight does not present changes between genotypes nor intervention groups (Figure R20D).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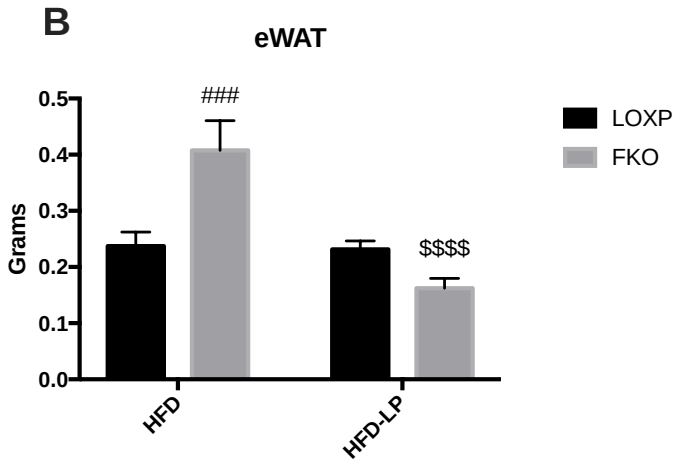
FKO


B
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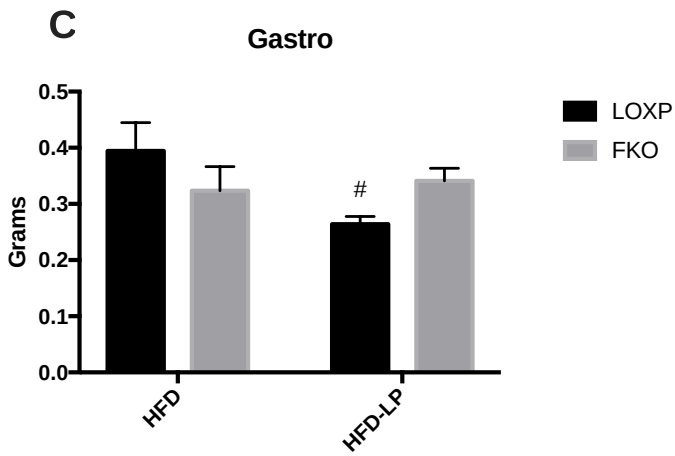
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Gastro
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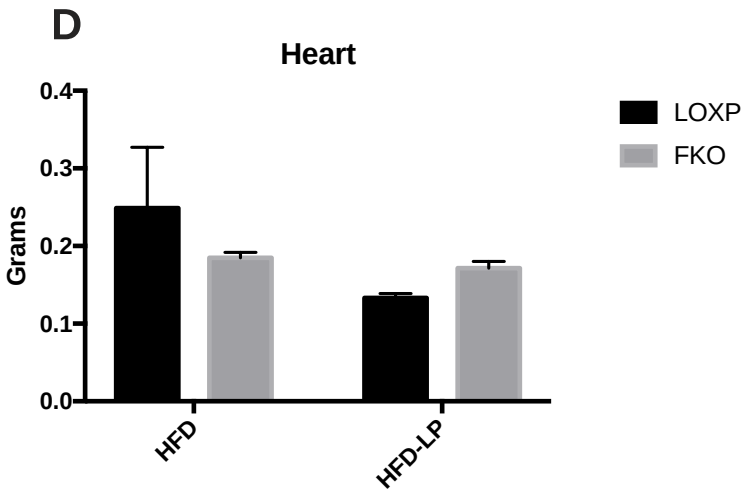


Figure R20. Tissues weights of LoxP and LFKO mice fed with an HFD or an HFD-LP after the 10-week nutritional intervention. Subcutaneous white adipose tissue (scWAT) (A), epididymal white adipose tissue (eWAT) (B), gastrocnemius (Gastro) (C), and heart (D) weight gain of mice fed with an HFD or an HFD-LP for 10-weeks. Tissues weight was measured immediately after sacrifice. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs HFD LoxP. $p<0.05; $$p<0.01; $$$p<0.001 vs HFD LFKO. *p<0.05; **p<0.01; ***p<0.001 vs HFD-LP LoxP.

HFD-LP intervention induced Ucp1 in scWAT and BAT

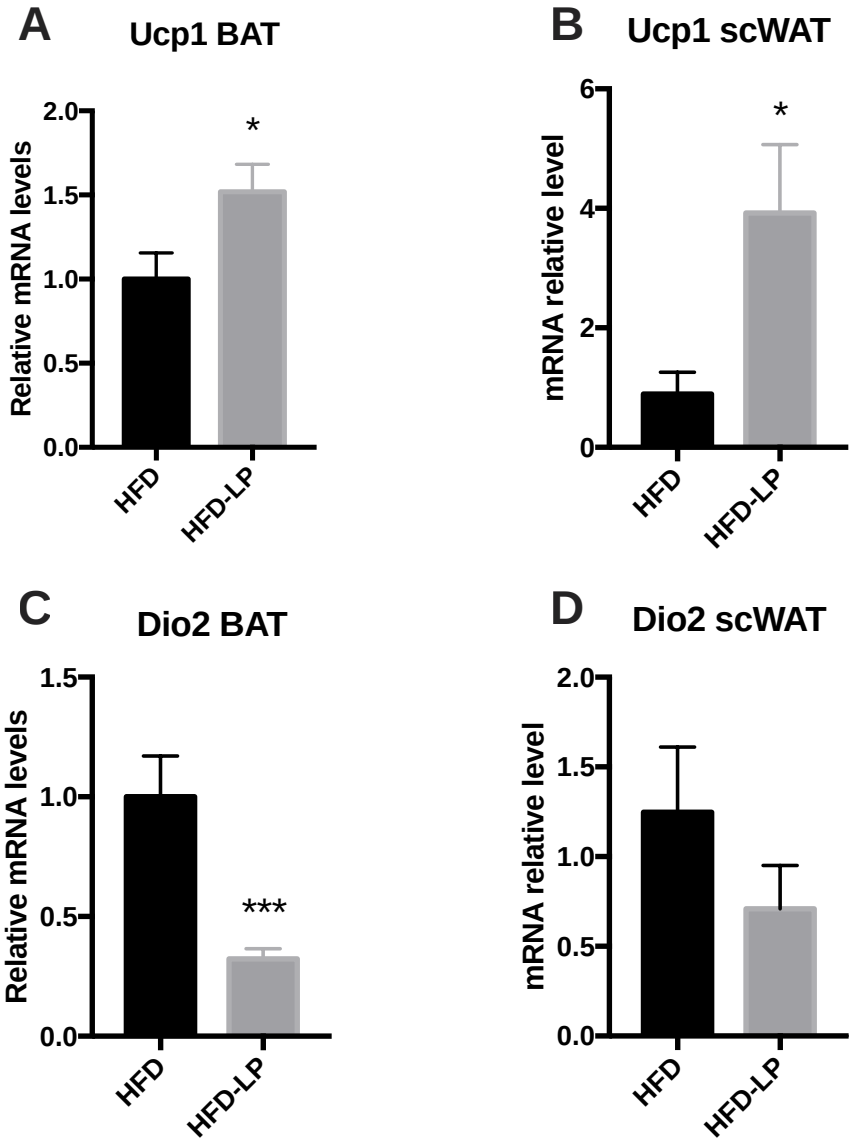
Results published by our laboratory demonstrated that FGF21 is induced by amino acid deprivation and mediates the induction of UCP1 and other thermogenic genes in fat tissues [67]. UCP1 expression defines thermogenic brown adipocytes by its ability to dissipate heat by uncoupling proton transport from ATP synthesis [150]. FGF21 is a potent stimulator of the thermogenic capacity by engaging the receptor complex comprised of FGFR1 and the requisite coreceptor KLB [172].

To determine the thermogenic capacity in adipose tissue mediated by protein restriction within an HFD, we analyzed the gene expression of *Ucp1* and iodothyronine deiodinase 2 (*Dio2*) in scWAT and BAT of Loxp mice fed with an HFD or an HFD-LP.

Dio2 gene encodes the type 2 deiodinase that catalyzes the conversion of the prohormone thyroxine (T4) to the active 3,3´5-triiodothyronine (T3). Adequate quantities of thyroid hormones are required for the maintenance of basal energy expenditure. When Dio2 is activated in BAT, the levels of T3 increase, and there is a saturation of thyroid hormone receptor that causes intracellular thyrotoxicosis, specifically in this tissue. This activation of the T3 signaling pathway increases BAT's adrenergic responsiveness and induces thermogenesis [173].

Results showed an increased expression of *Ucp1* in BAT and scWAT (Figure R21A, 21B), thus indicating that LoxP mice, after an HFD-LP, showed an induction of a thermogenic program in these tissues. By contrast, under an HFD-LP diet, the *Dio2* mRNA levels are markedly reduced in BAT, and shows a clear tendency in scWAT of LoxP mice (Figure R21C, 21D). *Cpt1b* and *Prmd16* gene expression analysis in scWAT did not present changes in mRNA expression after an HFD-LP intervention in LoxP mice compared to HFD-fed mice, suggesting that Ucp1 mediates the effects of protein restriction over thermogenesis in scWAT during HFD-induced obesity. Gene expression analysis in LFKO should be measured for

more information about FGF21 effects and thermogenesis in these tissues.



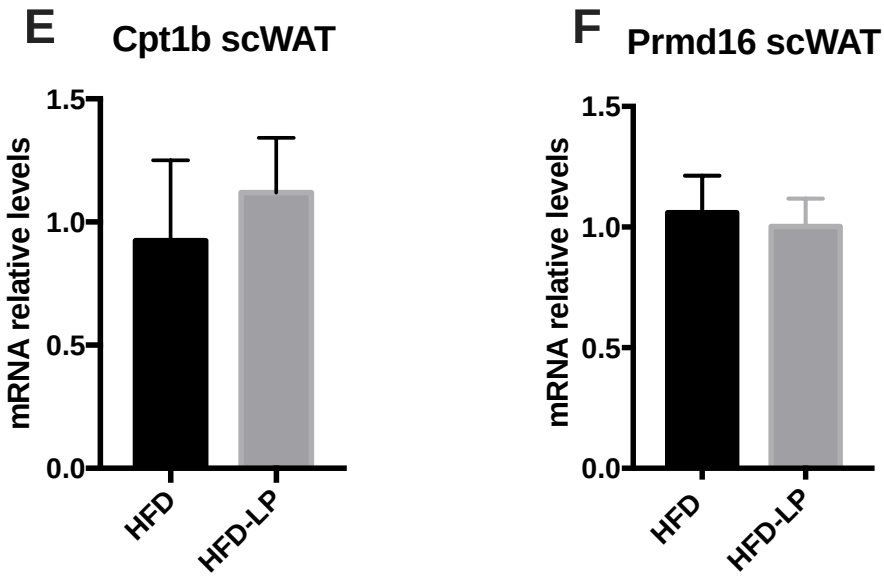
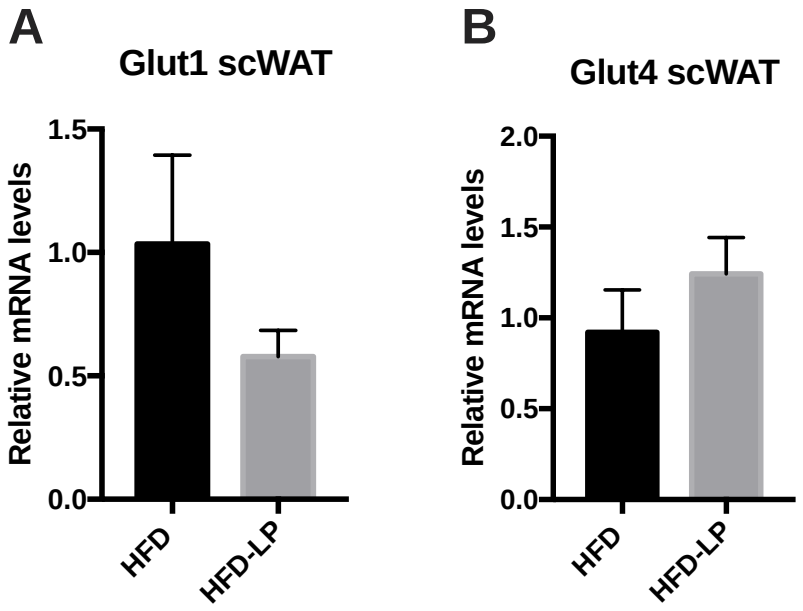


Figure R21. Gene expression analysis of genes involved in thermogenesis. A low protein intake within an HFD induces the expression of Ucp1 relative mRNA levels in BAT (A) and scWAT (B). Dio2 expression in BAT (C) and a marked downregulation in scWAT (D). mRNA levels of *Cpt1b* (E) and *Prmd16* (F) in scWAT. Bars represent the fold induction in the mRNA levels versus the Loxp HFD mice that are considered the control group, which produces an arbitrary value of 1. Data are presented as the mean ± SEM. *p<0.05, ** p<0.01, ***p< 0.001 vs Loxp HFD.

The expression of glucose receptors is not affected by protein restriction in adipose tissue

It has been described that FGF21 LFKO mice have reduced expressions of GLUT4 in subcutaneous adipose tissues after an HFD induction [174]. To verify how protein restriction functions to improve glucose tolerance during HFD-induced obesity, we studied the main glucose receptors in adipose tissue of Loxp mice fed with an HFD or an HFD-LP.

We found that glucose receptors, *Glut1,* and *Glut4* in scWAT (Figure R22A, 22B) and BAT (Figure R22C, 22D) are not induced in mice after an HFD or an HFD-LP after the 10-week nutritional intervention. These results suggest that the upregulation of glucose transporters does not mediate the improved glucose tolerance found during a protein restriction intervention. This indicates that other mechanisms may be involved in a better response to glucose and insulin during a protein-restricted nutritional intervention.



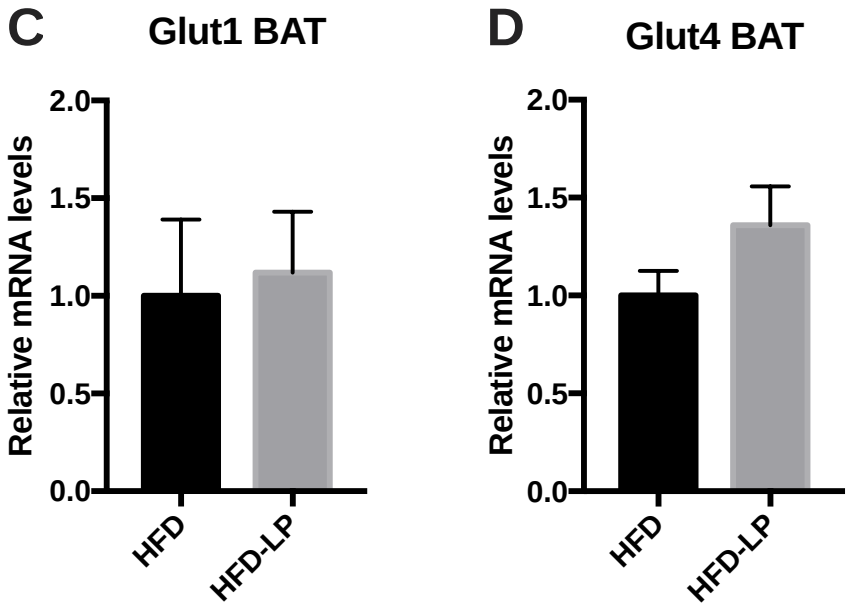


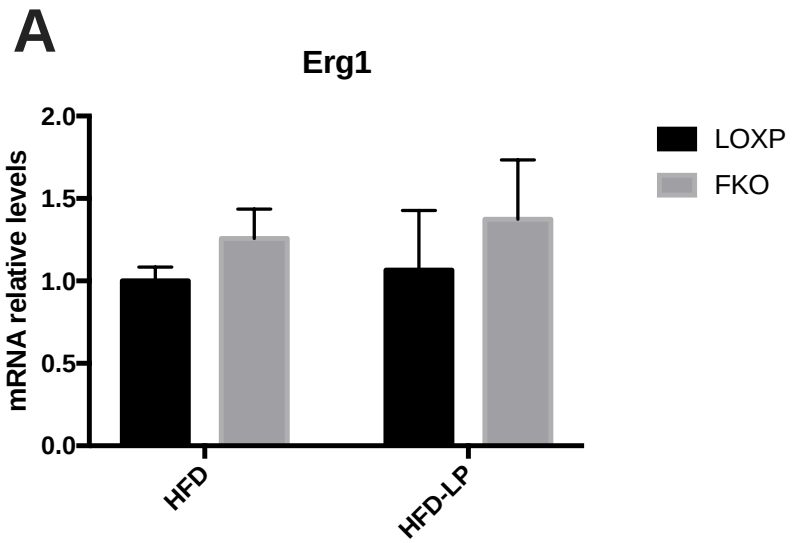
Figure R22. Gene expression analysis of genes involved in glucose transport in scWAT and BAT of Loxp and LFKO mice fed with an HFD or an HFD-LP. mRNA relative expression of Glut1 (A) and Glut4 (B) in scWAT; Glut 1 (C) and Glut 4 (D)mRNA levels in BAT.

FGF21 receptors in the brain are not induced by a HFD nor HFD within protein restriction

To understand the mechanisms involved in the metabolic response during protein restriction, we considered the brain's FGF21 signaling. Hill et al. recently published a study regarding the mechanisms involved in the FGF21 response during protein restriction and diet-induced obesity. The authors mentioned that FGF21 signaling in the brain is key to promoting a low protein diet's metabolic effects within diet-induced obesity [13]. Deletion of the

FGF21 co-receptor KLB from the brain causes and impairment in the metabolic response to a low protein diet, thus, indicating that the FGF21 actions thought KLB in the brain are needed to promote the homeostatic response.

Therefore, we measured the mRNA levels of *Egr1* (Figure R23A), and FGF21 target gene, and *FgfR1* (Figure R23B) in the hypothalamus of LoxP and LFKO mice fed with an HFD or an HFD-LP. The results showed no differences in the induction of both genes when fed with a restricted protein diet.



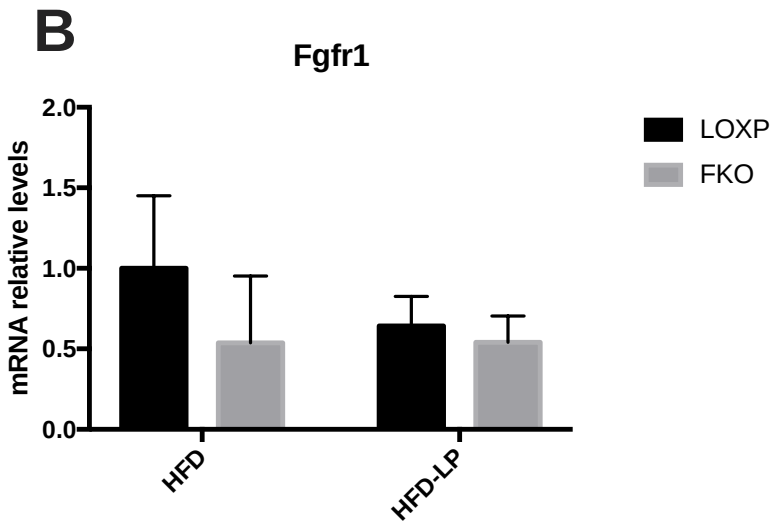


Figure R23. Gene expression analysis of genes involved in FGF21 signaling in the hypothalamus. A low protein intake within an HFD does not exert effects in ERG1 (A) and FGFR1 (B) mRNA levels in the hypothalamus in LFKO and LoxP mice. Bars represent the fold induction in the mRNA levels versus the Loxp HFD mice that are considered the control group, which produces an arbitrary value of 1.

Chapter 3

Role of FGF21 in the metabolic response to fasting

FGF21 has been described as the missing link to fasting [175,176]. Hepatic Fgf21 expression is induced directly by PPARα in response to fasting, and its expression has also been associated with specific metabolic pathways involved in the control of energy homeostasis during nutrient deprivation. It has been described as crosstalk between FGF21, insulin, and glucagon [177]. In the liver, FGF21 activates fatty acid oxidation, ketogenesis, and gluconeogenesis, thus triggering a state that mimics the metabolic response to prolonged fasting.

These observations suggest that FGF21 would play an essential role in regulating energy metabolism during late fasting. However, the specific role of FGF21 in this adaptive state of nutrient deprivation is not yet clear. To elucidate the physiological roles of Fgf21, we analyzed the metabolic profile of Fgf21 KO mice during 17h fasting.

Hepatic FGF21 expression is induced after 17h fasting

LoxP and LFKO mice were sacrificed after 17h of fasting and its metabolic profile was analyzed and compared with LoxP and LFKO mice in fed state. Firstly, the mRNA levels of hepatic Fgf21 was measured to confirm that its expression was induced by the 17h of fasting in LoxP mice and no induction was detected in LFKO (Figure

R24). As it is shown in Figure R24, the hepatic expression of Fgf21 was greatly induced by 17h.

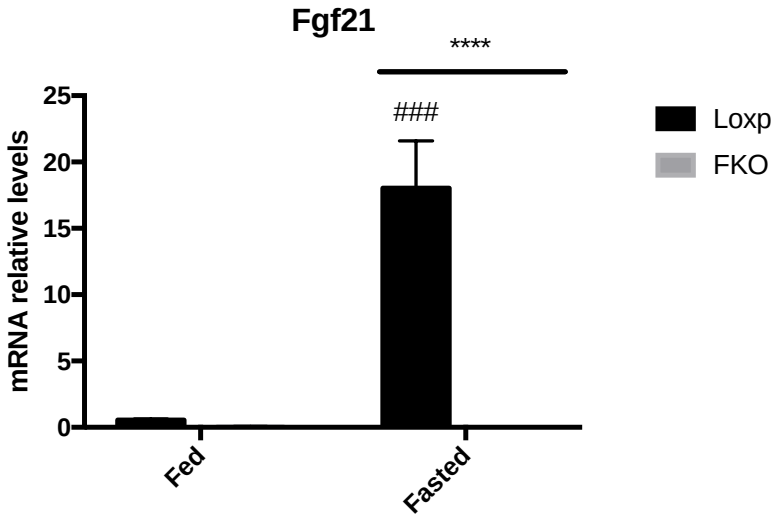


Figure R24. Fgf21 mRNA expression levels in the liver of LoxP and LFKO mice in fed or fasted state.The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs Fed LoxP. *p<0.05; **p<0.01; ***p<0.001 vs Fasted LoxP.

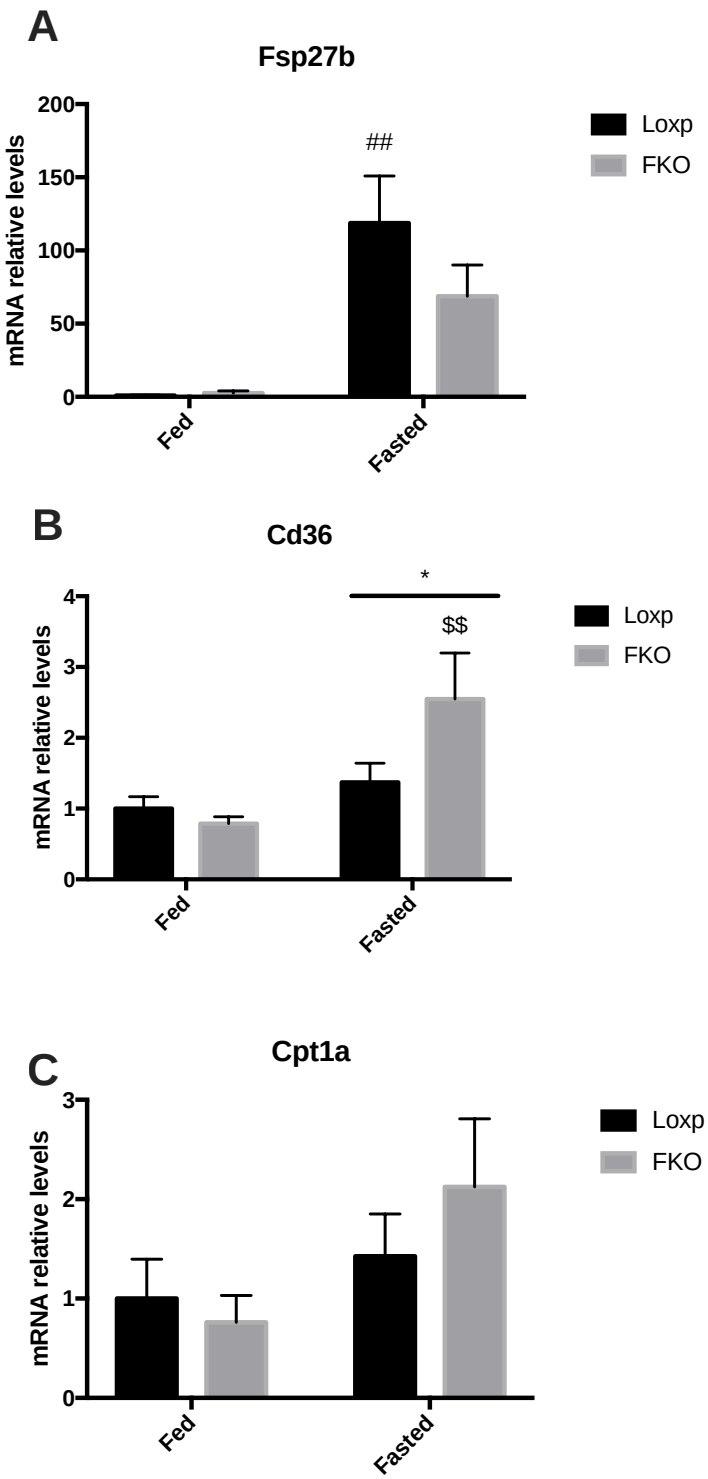
As FGF21 is widely recognized as a potent regulator of energy homeostasis, we analyzed its effects over genes involved in lipid metabolism, fatty acid oxidation and lipid droplet formation in the liver or LoxP and LFKO mice in fed or 17h fasted state.

Fsp27b **and** ***Cpt1*** **regulation in fasted state depends in part in the effects of hepatic FGF21 in the liver**

FSP27 is a lipid droplet-associated protein that promotes LD growth and triglyceride storage in white adipocytes, liver, and muscle. Adipocytes primarily express FSP27α isoform, and FSP27β tends to

be expressed more in the liver [164]. Additionally, a specific protein regulates each isoform; peroxisome proliferator-activated receptor gamma (PPARγ) regulates adipose-specific FSP27α, and cyclic-AMP response element-binding protein (CREB) regulates FSP27β [163]. The expression of the hepatic isoform of FSP27, the *Fsp27b,* was analyzed, and results showed that *Fsp27b* mRNA levels were markedly induced after 17h of fasting in LoxP mice. This induction was partially abrogated in LFKO mice, pointing out the putative role of FGF21 in this induction (Figure R25A).

The *Cd36* and *Cpt1a* mRNA levels were analyzed in the liver of LoxP and LFKO mice in the fed or 17h-fasted state. Results showed that *Cd36* and *Cpt1a* mRNA levels in the liver during fed state were not affected by the absence of hepatic FGF21 (Figure R25B, R25C). During fasting, no changes in the expression of *Cd36 or Cpt1a* were reported in the LoxP mice (Figure R25B, R25C). However, LFKO mice presented a marked induction in *Cd36* after 17h fasting compared to the LoxP mice fasted (Figure R25B), indicating that hepatic FGF21 contributed to the regulation of this gene during the fasted state. *Cpt1a* levels during fasting were not affected, indicating that hepatic FGF21 does not mediate this response during fed or fasted state (Figure 25C).


A
Fsp27b
mRNA relative levels
200
150
100
50
0
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Loxp
FKO
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Cd36
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mRNA relative levels
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Loxp
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Fed
Fasted

Figure R25. mRNA expression of genes involved in lipid metabolism in the liver of LoxP and LFKO mice in fed or fasted state. *Fsp27b* mRNA levels are induced in the fasted state in a manner partially dependent on FGF21 (A). *Cd36* (B) and *Cpt1a* (C) are not affected by 17h fasting. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs Fed LoxP. $p<0.05; $$p<0.01; $$$p<0.001 vs Fed LFKO. *p<0.05; **p<0.01; ***p<0.001 vs Fasted LoxP.

Triglycerides accumulation in the liver is not dependent on hepatic FGF21

During fasting there is a physiological accumulation of TG in the liver as a supply of fatty acids for beta-oxidation. To confirm whether hepatic triglyceride accumulation in fasted state depends on the hepatic FGF21, liver triglyceride levels were quantified in LoxP and LFKO mice (Figure R26). As expected, the results indicated that triglycerides storage is increased in the liver in response to the arrival of fatty acids coming from adipose tissue in the 17h fasted state. However, this accumulation is not dependent on hepatic FGF21 as the values were the same in LoxP than in LFKO mice.

Altogether, these results suggested that the main mechanisms to maintain energy homeostasis in the liver during 17h fasting is lipid droplet formation by *Fsp27b* induction, TAG storage, and *Fgf21* induction. During this nutritional deprivation state, FGF21 is markedly induced and plays a role regulating in fatty acid transport into the hepatocytes coming from WAT lipolysis.

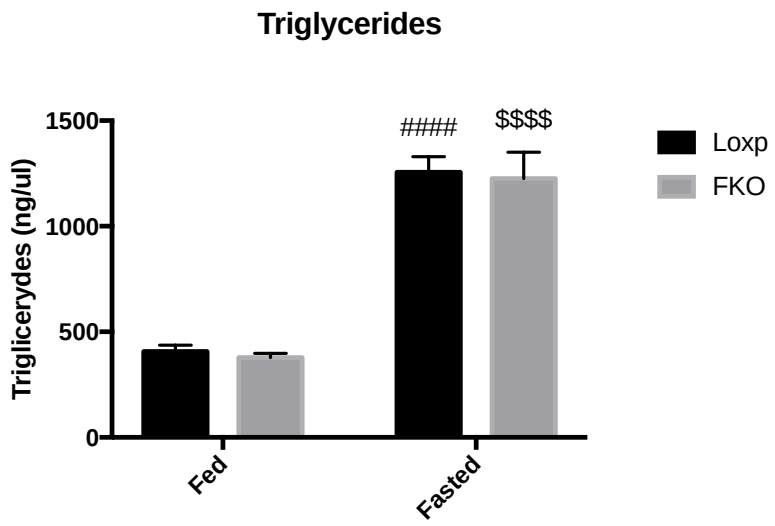


Figure R26. Triglyceride quantification in the liver of LoxP and LFKO mice in fed or fasted state. The accumulation of triglycerides in the liver increases in the starvation state and is independent of FGF21. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs Fed LoxP. $p<0.05; $$p<0.01; $$$p<0.001 vs Fed LFKO.

Gluconeogenesis is dependent on the expression of hepatic FGF21 during fasting

During middle fasting, hepatic gluconeogenesis is the primary source of endogenous glucose. Gluconeogenesis is a metabolic pathway involved in glucose formation, using as precursors no glycosidic compounds such as amino acids and lipids and is an essential pathway in regulating blood glucose homeostasis [178].

To determine the role of FGF21 in this metabolic pathway, we analyzed the expression of two key enzymes of gluconeogenesis: the *G6pase* and *Pepck* (Figure R27). Our results showed that

mRNA levels of both gluconeogenic genes are induced in the liver during the fasted state. This induction is partially impaired in the LFKO mice, thus indicating the role of FGF21 in this effect.

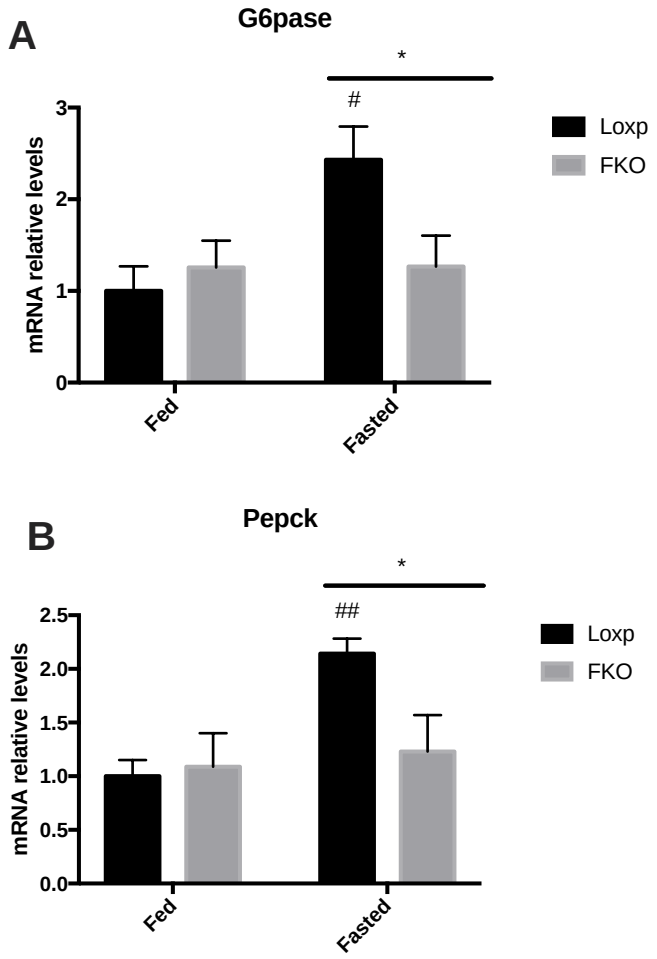


Figure R27. Expression levels of genes involved in fatty acid uptake, oxidation, and hepatic gluconeogenesis. The expression of G6Pase (A), Pepck (B) was analyzed by means of qRT-PCR in the liver of LFKO and LoxP mice in the fasted and fed state. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs Fed LoxP. *p<0.05; **p<0.01; ***p<0.001 vs Fasted LoxP.

Ketogenesis is not dependent on hepatic FGF21 during fasting

One of the essential metabolic pathways in response to fasting circulating glucose deficiency is the ketogenesis pathway that produces ketone bodies (acetoacetic acid and 3-hydroxybutyric acid) [179].

To determine the regulatory effects of FGF21 on ketogenesis, we analyzed the expression of the *mitochondrial hydroxymethylglutaryl-CoA synthase* (*Hmgcs2*), a key enzyme in the formation of ketone bodies. Our results indicated that *Hgmcs2* expression tended to increase in the fasted state, but this induction did not depend on hepatic FGF21 as it is similar in LoxP animals and LFKO (Figure R28A). To confirm this data, serum β-hydroxybutyrate levels were measured. Results showed that ketone bodies' concentration increased significantly after 17 hours of fasting in both LoxP and LFKO mice, indicating that the presence of FGF21 is not required for this induction (Figure R28B).

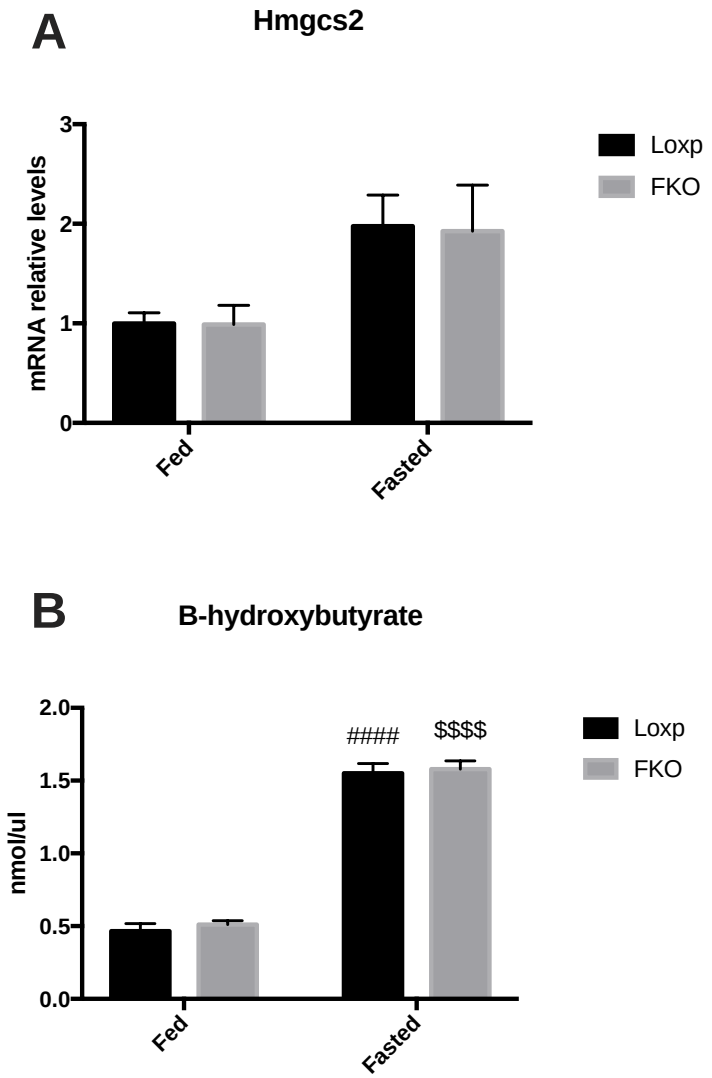


Figure R28. Hepatic expression of Hmgcs2 (A) and B-hydroxybutyrate levels in liver (B) of LFKO and LoxP mice in fed or fasted state. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs Fed LoxP. $p<0.05; $$p<0.01; $$$p<0.001 vs Fed LFKO.

CREBH is induced during fasting in an FGF21 dependent pathway

The gene expression pattern of 17h-fasted mice showed a clear differential profile regarding the role of FGF21 in its regulation. *Fsp27b, G6Pase,* and *Pepck* are induced in the fasted state in an FGF-dependent way, and all of them are target genes of the Cyclic AMP-responsive element-binding protein 3-like 3, hepatocyte-specific (CREBH).

CREBH is a liver-specific, endoplasmic reticulum (ER)-localized transcription factor of the CREB/ATF family. Some authors defined CREBH as a stress-inducible transcriptional activator induced by metabolic stress [180,181] and fasting. CREBH acetylation in mouse livers appear in a time-dependent manner, and this event is critical for CREBH transcriptional activity in regulating hepatic lipid homeostasis [182].

To determine if CREBH mediates the response between FGF21 and *Fsp27b* and gluconeogenic genes induction after a 17h fasting, and we analyzed the *Crebh* expression in the liver of LoxP and LFKO mice (Figure R29). Results indicated that *Crebh* mRNA levels are overexpressed during 17h fasting, and its induction is dependent on the presence of hepatic FGF21.

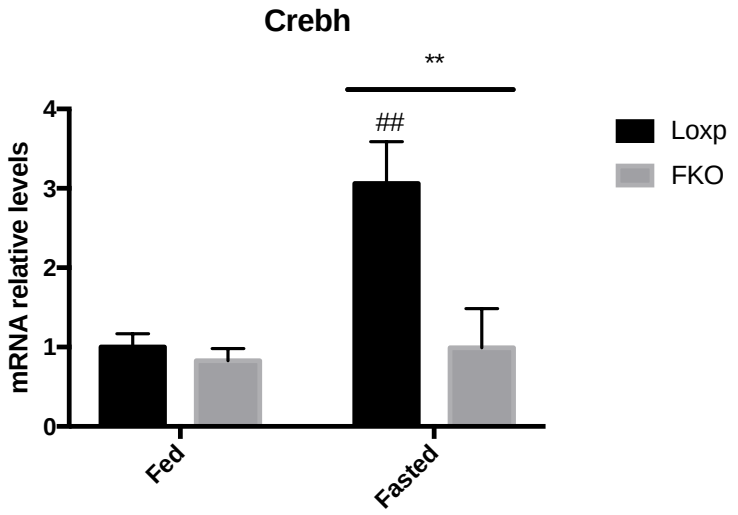


Figure R29. Hepatic mRNA levels of *Crebh*. The graph represents the mean +/- SEM. #p<0.05; ##p<0.01; ###p<0.001 vs Fed LoxP. *p<0.05; **p<0.01; ***p<0.001 vs Fasted LoxP.

Discussion

The effects of FGF21 are subtle under normal conditions but increase significantly under metabolic and nutritional challenges, reinforcing its key role in restoring metabolic homeostasis. Our results demonstrated that different metabolic stress conditions such as ER stress caused by TM administration, a prolonged energy overload, protein restriction, and fasting could cause a dramatic induction of hepatic FGF21 levels.

FGF21 in ER stress caused by TM

Obesity and hepatic steatosis have been described as pathological conditions that display an increase in cellular stress. The liver is susceptible to ER stress. Numerous studies had reported the crucial role of ER stress and UPR signaling pathways in the pathogenesis of liver diseases [183,184], which may promote homeostasis maintenance or cell death when homeostasis is not archived. These mechanisms may underlie the elevated FGF21 levels observed in patients with NAFLD [185].

TM is a ER stress inducer [138]. It inhibits the phosphorylation of protein kinase B (Akt) leading to a disruption of insulin, glucose, and triglycerides metabolism. Also, it has been reported that TM reduces apolipoprotein expression, lipoprotein secretion, glucose production, hepatocyte apoptosis and it can inhibit hepatic gluconeogenesis [76] leading to an impairment in glucose and lipid homeostasis in the liver.

Our laboratory recently published a low leucine diet mediated by GNC/ATF4 induced FGF21 levels [186]. GNC2 phosphorylates and inactivate eIF2a, which repress protein translation except for the ones produced to counteract amino acid starvation [62]. Also, ATF4 contributes to the cellular response to diverse stressors such as ER stress induced by TM administration. The UPR is activated as a response to counteract misfolded proteins in the ER lumen, and it causes PERK phosphorylation of eIF2a, leading to the activation of ATF4 which contributes to FGF21induction [5].

Our results demonstrate that *Fgf21* is highly induced after TM injection and contributed to the UPR mediating, at least in part the induction of *Bip* and *Chop*. BiP is a mayor ER chaperone, and it acts as a primary sensor in the induction of the UPR. Recent findings published by Kopp et al. demonstrated that the interaction of BiP with IRE1 and PERK produces BiP change from the chaperone cycle to an ER stress sensor cycle, by preventing the binding of its co-chaperones [187]. Our findings suggest that *Bip* expression depends on hepatic FGF21, demonstrating its role in the cell response to ER stress contributing to the UPR.

Our results also showed that FGF21 contributes at least in part to Chop induction, which is a protein involved in ER stress-induced apoptosis, regulation of genes that encode proteins involved in proliferation, expression, and energy metabolism [188]. However, ATF4 also contributes to FGF21 [5] and CHOP induction, which subsequently downregulates anti-apoptotic BCL-2, stimulates pro-

apoptotic Bim, and contributes to apoptosis [189]. Thus, FGF21 is induced by ATF4 as part of the UPR response, and established the FGF21 role in the protein quality control of the ER and its contribution to the activation of the ER-transmembrane signaling molecules [185].

The effects of endogenous FGF21 over ER stress and TG storage in the liver caused by TM was not well understood. Some articles had reported that the administration of recombinant FGF21 in mice alleviated TM-induced liver steatosis [190,191]. The present work results provide a new approach to endogenous FGF21 actions over lipid metabolism during acute metabolic stress caused by TM in the liver.

Acute storage of TG in the liver, caused by TM administration, produced an increased *Dgat1* expression in the liver, a key enzyme involved in TG synthesis. These data coincide with previously published data reporting the effects of TM over hepatic triglyceride accumulation [137,140,192]. Feng et al. reported that after 24h mice injected with TM presented a significantly yellowish color, ER stress, hepatic TG were remarkably increased, and apolipoprotein B100 expression was suppressed [137].

However, regarding the effects of FGF21 over TG storage caused by TM administration, our results show that endogenous FGF21 is not involved in this hepatic TG accumulation nor its amelioration. Loxp and FKO mice exhibited similar values in the TG quantification

or *Dgat1* mRNA levels in the liver. These data indicate that the endogenous FGF21 cannot ameliorate the TG accumulation caused by TM injection. Further studies are needed to evaluate the FGF21 signaling after the TM administration. The levels of receptors and downstream signaling molecules should be evaluated to identify why the increment of FGF21 caused by TM treatment cannot reduce the TM-induced hepatic steatosis.

Similarly, the *Fgf21* mRNA levels after TM injection correlated negatively with the changes observed in the ALT and AST levels, biochemical parameters related to liver injury. An association between *Fgf21* levels an improved AST:ALT ratio is consistent with the concept that FGF21 has a role in the prevention of progressive disfunction and cell death in the liver. These results agree with the ones published by Escoté et al., which demonstrated a negative relationship between FGF21 and AST/ALT ratio in serum samples from overweight/obese women [193]. These results agree with other studies published by Maruyama et al., were FGF21-deficiency caused severe ER stress in the liver and exacerbated TM-induced ER stress target gene expression respect to the wild-type mice [191]. All in this together suggest that FGF21 may be involved in preventing liver injury and may have protective effects over oxidative stress and chronic inflammation.

Protein restriction to counteract diet-induced obesity: the role of hepatic FGF21

Obesity is one of the major causes of NAFLD. Obesity-induced insulin resistance is also linked to NAFLD. Insulin resistance happens when insulin-sensitive tissues lose insulin response, leading to an impairment of insulin-mediated glucose uptake. One of the mechanisms that explain insulin resistance in the context of overnutrition is adipose tissue disfunction/lipotoxicity, which includes inflammation, mitochondrial dysfunction, ER stress, and hyperinsulinemia [119].

In obesity, the unmitigated stress and impaired fatty acid oxidation caused by a prolonged energy overload lead to a severely dysfunctional WAT [119]. Adipocytes expand in cell size and number to compensate for the necessity of increased lipid storage. Adipocytes eventually reach a limit due to tissue expansion limitations, leading to an inflammatory program activation. When WAT capacity is overwhelmed, additional caloric overload stimulates ectopic fat deposition in other tissues that regulate glucose homeostasis, such as the liver, muscle, or pancreas [194,195]. This mechanism is recognized as lipotoxicity. Ectopic fat accumulation in the pancreas contributes to B-cell dysfunction and systemic insulin resistance [119].

Obesity is a state in which circulating levels of FGF21 are elevated, possibly as a response to overcome excess energy income and

triglyceride accumulation [196]. However, in an obesity state, endogenous FGF21 levels appear to be ineffective [197].

Previously published works had demonstrated that protein restriction is an efficient way to increase FGF21 production [14,158] and that, at least in part, the metabolic effects of amino acid and protein-deficient diets go through the induction of FGF21 [13]. In the liver, leucine deprivation caused induction of *Fgf21* expression and inhibition in lipogenic genes activity such as *Fas* and *Srebp1c* expression. Also, leucine deprivation, mediated by FGF21, mobilized lipid stores in adipose tissue [14]. Also, Perez-Martí et al. demonstrated that a LPD increased FGF21 production by inducing its liver expression, and it was associated with lower body weight in mice. Browning of WAT was also reported after an LPD intervention by induction in *Ucp1* expression in scWAT in mice [158]. Taking together all these data, the protein restriction may be considered as a new therapeutic approach to prevent the metabolic disturbances caused by diet-induced obesity.

Our results demonstrate that protein restriction within a HFD induced hepatic FGF21. Similar results were published by Tanaka et al. mentioned that FGF21 is elevated in the early stage of NASH to minimize hepatic lipid accumulation and counteract ER stress [198]. Primary hepatocytes treated with palmitic acid showed elevated levels of *Fgf21* mRNA. This induction of the *Fgf21* expression is not dependent on the PPARa signaling but seems to

be related to the lipid-induced ER stress that enhances *Fgf21* expression in the liver [198].

On the other side, it has been described that enhanced FGF21 levels in NAFLD patients are caused by dysfunctional PPARa signaling [199]. Mice with NAFLD showed an increased response to PPARa agonists leading to higher FGF21 expression than the non-NAFLD mice [200] In this case, the elevated plasma levels of FGF21 were associated with lower expression of KLB, suggesting an FGF21 resistance [200]. During NAFLD, PPARa is activated by intrahepatic fatty acids [201], and prolonged activation of this nuclear receptor leads to increased FGF21 levels[200].

Our results indicate that FGF21 mediated the effects of protein restriction within a HFD by preventing weight gain progression, liver weight gain and reducing fat storage in the liver. Similar results were published by Laeger et al. [39]. This publication showed that protein restriction alters food intake, energy expenditure, and body weight gain in an FGF21-dependent way, as FGF21-deficient animals did not exhibit these effects in response to a LP diet [66]. Moreover, a study published by Owen et al. mentioned that FGF21 acts centrally by stimulating the sympathetic nervous system -BAT axis, causing effects on energy expenditure and body weight in obese mice [39].

After 10-week of protein restriction mice, our experimental approach showed a tendency to reduce TG accumulation in the liver and a lower liver weight gain mediated by endogenous hepatic FGF21.

Zhu et al found similar results. in an experiment with Monosodium L-glutamate (MSG)-induced obese mice. In this case, the results showed that FGF21 significantly reduced serum triglycerides and reversed hepatic steatosis by increasing the expression of several proteins related to autophagy of lipid droplets [202]. Autophagy has been described as a critical mechanism of lipid mobilization in hepatocytes; its inhibition is linked to NAFLD and insulin resistance [202].

Similarly, the pharmacological administration of FGF21 reduces TG accumulation in the liver. Jimenez et al. demonstrated that a single administration of adeno-associated viral vector (AAV) vectors encoding FGF21 enabled a long-lasting increase in FGF21 levels leading to a reversal of hepatic steatosis, inflammation, and fibrosis in HFD-fed mouse and ob/ob mouse [203]. These results are also corroborated by a clinical trial with patients diagnosed with NASH that were treated during 16-weeks with a recombinant human analog of FGF21. The results showed that FGF21 analog significantly decreased fat deposition in the liver and was associated with a reduction in fibrosis and hepatic injury [204]. Altogether these data highlight that pharmacological and endogenous FGF21 are potential candidates in treating liver pathologies as NAFLD and NASH.

The dysregulation of the flow of fatty acids from adipose tissue to the liver has been associated with NAFLD and insulin resistance [161,166]. The lipid homeostasis is a crucial point in the

pathogenesis of NAFLD. Dysregulation of lipogenic genes expression is widely associated with NAFLD pathogenesis. Globally our results show changes in lipid and glucose metabolism-related to protein restriction and/or FGF21 signaling within HFD.

Our results demonstrated that a restricted protein intervention, mediated by hepatic FGF21, attenuates *Cd36* expression. The hepatic FGF21 deficient mice with the same dietetic intervention presented a *Cd36* overexpression, which had been linked to fatty liver progression during energy overload by the upregulation of fatty acid uptake by the hepatocytes. Wilson et al. showed that a hepatocyte-specific CD36 deletion prevented lipid storage and improved inflammatory markers, ALT and AST, in HFD-induced obese mice [205]. These effects suggest a protective FGF21 effect over fatty liver progression by downregulating hepatic *Cd36* expression.

Besides its effects on de novo lipogenesis, FGF21 mediated lipid droplet formation by controlling Fsp27b expression. FSP27b has been identified as a key molecule involved in fatty acid storage and NAFLD pathogenesis [165]. A HFD intervention does not affect hepatic Fsp27b expression in LoxP and FKO mice. However, our data show that FGF21 downregulates *Fsp27b* expression in obese mice's liver when fed with a protein-restricted diet. Hepatic FGF21-deficient mice presented an upregulation of hepatic *Fsp27b* expression after a HFD-LP intervention. In this case, the *Fsp27b* upregulation may contribute to lipid droplet formation in the liver,

contributing to hepatic steatosis progression by previously published data. These results agree with the ones published by Xu et al., which demonstrated that hepatic steatosis in ob/ob mice is promoted by FSP27, as the knockdown of FSP27 in ob/ob mouse liver ameliorated the hepatic steatosis [165]. All in this together suggests that protein restriction, mediated by FGF21, prevented lipid accumulation in the liver by suppressing *Fsp27b* expression.

Srebp1c has been related to NAFLD development because of its effects regulating the expression of lipogenic genes such as FAS and acetyl-CoA carboxylase (ACC) [206]. The overexpression and maturation of SREBP1c lead to an increase of de novo lipogenesis that contributed to the accumulation of fatty acid. A protein restriction intervention downregulates the Srebp1c expression in diet-induced obese mice in an FGF21-independent manner in our experimental approach. This reduction of the *Srebp1c* levels would lead to a reduced TG storage in this tissue. Zhu et al. described that in mice with calorie restriction, the insulin/IGF-1 signaling pathway's role was related to a reduced hepatic SREBP-1c and FAS levels, suggesting that a dysfunctional insulin receptor may contribute to an altered expression of genes involved in the lipid metabolism [207]. As it is showed in Figure R12, our diet-induced obese mice exhibited insulin resistance suggesting an impairment in insulin signaling and SREBP1c regulation. This impairment is ameliorated in HFD-LP-fed mice independently of hepatic FGF21. These data suggest that the regulation of SREBP1c activity by protein restriction is not dependent on the FGF21 signaling and may be related to the

improvement of the insulin signaling observed in HFD-LP-fed mice (Figure R17C).

Besides the impairment of lipid homeostasis, the dysregulation of glucose metabolism has also been implied in NAFLD pathogenesis. The stimulation of fatty acids oxidation is necessary for the endergonic steps of gluconeogenesis. It has been described that a dysregulation of the activity of the gluconeogenic gene is related to insulin resistance and the progression of NAFLD. Song et al. described that fat intake overload stimulates alanine gluconeogenesis via an increase of the fructose-1,6 bisphosphate protein levels in Wistar rats fed a HFD (60% fat) [208]. However, our results showed that after a 10-week HFD intervention (45% fat) with or without protein restriction, gluconeogenic genes were not altered in obese mice.

The improvement of insulin resistance has been related to adipose tissue depots' changes and energy expenditure capacity by some authors with different animal models of obesity and insulin resistance. Rats with type 2 diabetes and obesity fed with a low protein diet demonstrated that protein restriction prevented diabetic status progression. These results were related to reducing fat weight, enhancing the FGF21 levels, and the induction of the UPC1 expression in BAT Kim et al. described that the improvement of insulin sensitivity is mediated, at least in part, by promoting the healthy specific expansion of subcutaneous fat in C57BL/6 mice.

Their results suggest that subcutaneous adipogenesis is associated with measures of insulin sensitivity [209].

In our experimental approach, WAT depots (eWAT and scWAT) increased in FKO mice fed a HFD, but the role of FGF21 is blunted when protein restriction is within HFD. These data are partially different from those published by Li et al. Total FGF21 KO mice exhibited less subcutaneous mass and were more insulin-resistant when fed a HFD (45% fat) for 16 weeks [174]. The contradictory results may be due to the different animal models, in our experiment, a hepatic FGF21 KO model was used, and FGF21 effects may still be present by its induction by other tissues. Another essential factor to consider is the nutritional treatment duration, as Li et al. intervention was 16 weeks treatment compared to our ten weeks of intervention.

Besides WAT, liver weight increased under HFD in both genotypes and is only reduced in HFD-LP-fed LoxP mice. These data indicate that protein restriction and FGF21 affect fat distribution and lipid metabolism and, finally, insulin sensitivity.

The expression of glucose receptors in scWAT was analyzed as a possible mechanism contributing to glucose improvement during protein restriction in obese mice. Our findings show that glucose receptors in scWAT were not induced by protein restriction in obese mice, suggesting that insulin sensitivity improvement is not mediated by glucose uptake in this tissue. Besides glucose uptake, the

amelioration of insulin resistance could be related to increased fatty acid consumption. No changes in *Cpt1b* expression were observed in scWAT, but significant upregulation of Ucp1 mRNA levels was detected in scWAT and BAT of LoxP mice fed a HFD-LP. These data suggest that protein restriction within HFD increased in the thermogenic capacity of these tissues. It is essential to highlight that Dio2 is downregulated in BAT of these mice. Probably protein levels should be measured to confirm this result.

B3-adrenoreceptor stimulation has been related to an improvement of glucose levels by actions in adipose tissue. Kubo et al. published that in ob/ob mice with type 2 diabetes treated with a β3-adrenoreceptor activator, blood glucose was significantly lowered. In this case, neither glucose uptake nor *Ucp1* expression was changed in BAT and inguinal WAT. These results suggest that β3-adrenoreceptor contributes to glucose homeostasis but not mediated by glucose uptake In adipose tissue in ob/ob mice with type 2 diabetes [210]. Another study published by Granneman et al. showed that β3-adrenoreceptor stimulation induces metabolic adaptations such as increased metabolic rate, lipid oxidation, and body temperature that contributes to B3-adrenergic receptor-mediated thermogenesis, but in a UCP1-independent manner [211].

By contrast, other published data have related energy maintenance during a low protein intervention with UCP1 [10]. Our results show that a HFD-LP induces FGF21, and this may be related to the overexpression of UCP1 in BAT and scWAT (FKO data is still not

available). The upregulation of *Ucp1* expression may explain the improvement in glucose metabolism (insulin resistance and glucose tolerance) in mice with diet-induced obesity. Similarly, Kwon et al. reported that FGF21 mediates the UPC1 induction in adipose tissue, and UPC1-thermogenesis is required to improve glucose disposal and glucose tolerance [212]. Furthermore, FGF21 improves glucose clearance while preventing the fall in BAT temperature. Also, it has been described that a low protein diet enhances energy expenditure by inducing sympathetic flux via β-adrenergic receptor signaling to BAT, and a consequent upregulates UCP1 [213].

A recent study, with a similar methodology than the one we expose in this work, published by Hill et al. mentioned that a HFD with protein restriction improves glucose tolerance and lowers insulin levels in an FGF21-dependent manner thought its actions in the brain. Their results suggest that FGF21 and its signaling fully mediate the effects of overweight prevention and the improvement in insulin and glucose metabolism via KLB within the brain [214]. As shown in the results section in our experimental approach, the FGF21 signaling in the brain is not affected. These two approaches may be due to variations in the macronutrient composition, nutritional treatment duration, and the FGF21-KO model.

FGF21 during fasting: an adaptive signal

FGF21 is an essential regulator of the adaptive response to nutrient deprivation. FGF21 is highly induced during 17h fasting, and its

overexpression leads to an increase in gluconeogenic gene expression (*G6pase, Pepck*). These results agree with previously published data with FGF21 KO mice that exhibited severe hypoglycemia and an altered response in the hepatic gluconeogenesis under 24h fasting [215].

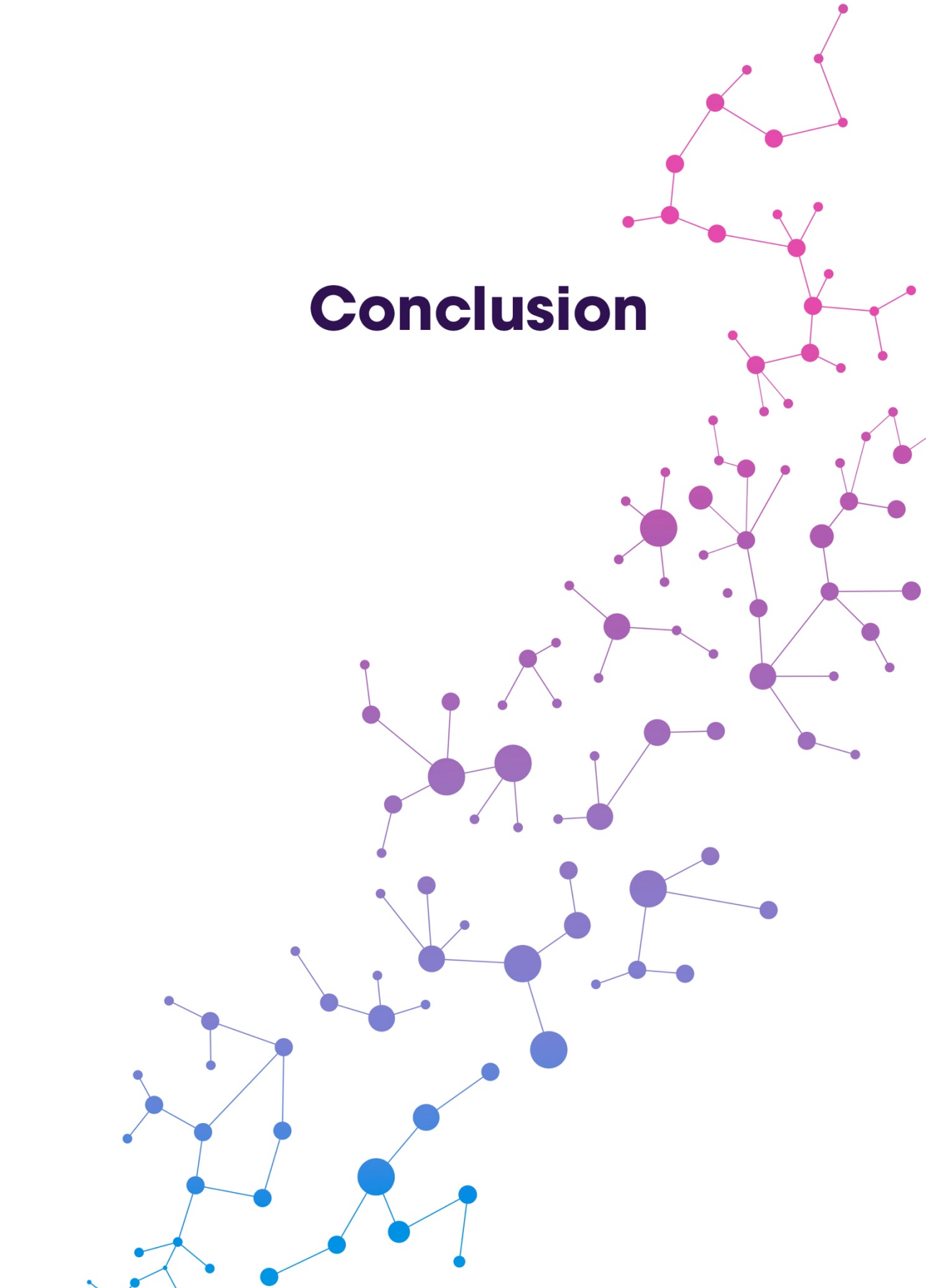
In physiological conditions, prolonged fasting induces FGF21, under the PPARa transactivation, then FGF21 gets into the brain to activate the hypothalamic-pituitary-adrenal axis (HPA) for the release of corticosterone and thus stimulate hepatic gluconeogenesis [37]. In our experimental approach, the expression of gluconeogenic genes: *G6Pase* and *Pepck* were not induced in FKO mice, demonstrating the impairment of gluconeogenic fasting response in these animals.

On the other hand, ketogenesis is induced in the fasted state but is not affected by *Fgf21* after 17h of fasting. These results are consistent with the concentration of B-Hydroxybutyrate in serum. Our results agreed with the ones published by Hotta et al., which described that FGF21 is not responsible for ketogenesis induction during fasting [216], as B-hydroxybutyrate levels significantly increased in FGF21 KO in comparison to the control mice, suggesting that FGF21 is not necessary for ketogenesis in the liver during the fasting state. In contrast, several authors had documented the ability of FGF21 to induce ketogenesis It has been demonstrated that FGF21-deficient mice fail to induce *Pgc-1α*, causing a failure in ketogenesis [34]. These contradictory results

could be due to the fasting experimental design, as in this experiment, a total of FGF21 KO mice were used with a 24h fasting intervention. In contrast, in our experimental design, hepatic specific FGF21 KO mice were used with a 17h fasting intervention.

What our results reveal is that specific fasting activated metabolic pathways are dependent on FGF21 signaling, and others do not. This may indicate that *Fgf21* would hold the key to initiating part of the prolonged fasting response by inducing hepatic gluconeogenesis and controlling *Fsp27b* expression, promoting metabolic change to later on activate the use of fatty acids as the main source of energy.

Conclusion



Role of FGF21 in the metabolic response to acute hepatic steatosis caused by tunicamycin administration

- Hepatic FGF21 does not prevent TG accumulation in the liver after 24h TM injection.

- Hepatic *Fgf21* ameliorates hepatic damage caused by TM by contributing to the activation of the UPR.

Effects of protein restriction mediated by FGF21 to counteract the metabolic outcomes of a high-fat diet

- Protein restriction effects on body weight within a HFD are dependent on the hepatic FGF21.

- Protein restriction in diet-induced obese mice counteracts the effects of HFD in the liver in part through the hepatic FGF21.

- Protein restriction within a HFD prevents liver weight gain, reduces hepatic steatosis, and downregulates the expression of genes involved in fatty acid transport and lipid droplet formation.

- Protein restriction via the hepatic FGF21 regulates lipid metabolism to ameliorate insulin resistance caused by HFD, corroborating its antidiabetic effects during obesity.

- A protein restriction within a HFD induces UCP1 expression in BAT and scWAT, suggesting an induction of the thermogenic program in adipose tissue. The role of FGF21 is that this response is still not elucidated.

Role of FGF21 in the metabolic response to fasting

- The metabolic response to fasting is partially dependent on FGF21 signaling. Gluconeogenesis induction is blunted in the absence of FGF21 but no ketogenesis.

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