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Is high fat diet induced FGF21 expression in epididymal adipose tissue affected by the addition of the rare sugar sweetener allulose?

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**UNIVERSITY OF
LIMERICK
OLLSCOIL LUIMNIGH**

NAME: GREG KERIN

ID: 18229301

SUPERVISOR: DR FABIANA HOFFMANN SARDA

DEPARTMENT: DEPT. OF BIOLOGICAL SCIENCES

PROJECT TITLE: "IS HIGH FAT DIET INDUCED FGF21

EXPRESSION IN EPIDIDYMAL ADIPOSE TISSUE

AFFECTED BY THE ADDITION OF THE RARE SUGAR

SWEETENER ALLULOSE?"

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**“Is high fat diet induced FGF21 expression in Epididymal
Adipose tissue affected by the addition of the rare sugar
sweetener Allulose?”**

Name: Greg Kerin

Student Number: 18229301

Abstract:

Background:

Fibroblast Growth Factor 21 (FGF21) is a pleiotropic peptide hormone which is expressed across a variety of different metabolically active tissues in mammals. FGF21 is part of the FGF19 subfamily of the FGF superfamily, and has a broad range of effects, depending on concentration, metabolic factors, and region in which it is expressed. FGF21 has been described as the “Starvation Hormone” as it is robustly expressed in hypo energetic diets and promotes several notable health benefits, such as glycaemic control, longevity and upregulating glucose neogenesis. FGF21 is also shown to be associated with negative health conditions and has been suggested for use as a biomarker, particularly for Liver conditions such as Non-Alcoholic Fatty Liver Disease (NAFLD) and mitochondrial disorders. Similarly, unhealthier diets, such as those higher in saturated fats, carbohydrates and free sugars are all shown to increase FGF21 levels. The objective of this study will be to analyse the RNA expression of FGF21, alongside the overall effect of Allulose supplementation to a high fat diet.

Methods:

C57/BL6 mice were exposed to an array of experimental diets in a previous study, Lean Diet (LD), High Fat Diet (HFD), Allulose (HFDa) and Fructose (HFDf). Serum expression levels of FGF21 amongst other metrics were measured during the course of this initial experiment. In this study, tissue samples from these test subjects were analysed. Liver sections were subjected to histological stains, and Epididymal Adipose Tissues from each group were analysed for FGF21 RNA expression. Quantitative Polymerase Chain Reaction (qPCR) was utilised to analyse this RNA expression, in conjunction with the housekeeping gene Peptidylprolyl Isomerase A (PPIA).

Results:

The addition of Allulose to the drinking water in HFD's induces a -1.73-fold change relative to HFDw FGF21 RNA expression levels, whereas the addition of Fructose induces a -0.23-fold change relative to HFDw FGF21 RNA expression levels. HFDw, relative to LDw, experiences a -0.4-fold expression change. Furthermore, weight gain was significantly reduced with the addition of Allulose to HFD, whereas the serum expression of FGF21 rose to near LDw levels. Lipid accumulation is also ameliorated with the addition of Allulose, decreasing TG content to near- LDw levels. OGTT metrics are also decreased, indicative that glycaemic control is improved overall, promoting insulin sensitivity.

Conclusion:

The addition of Allulose to High Fat Diets ameliorates the worse conditions associated with this diet: obesity, worsened glycaemic control, insulin insensitivity.

Allulose induced the serum expression of FGF21, which returned to near-normal levels when compared to HFD+ water diet. Moreover, Allulose decreased Epididymal Adipose Tissue FGF21 RNA expression, a 170% drop when compared to HFD. Despite localised downregulation, the increased serum expression appears to be linked with several metabolically beneficial effects, reducing the metabolic harm of a High Fat only diet would induce.

Acknowledgements:

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1.0 Introduction:

1.1 FGF21:

Fibroblast Growth Factor 21 (FGF21) is a member of the FGF superfamily, as a part of the FGF19 subfamily. FGF21 is a pleiotropic hormone, as it has an array of metabolic effects in humans and other mammals alike. FGF21 is involved in fatty acid oxidation (Annual Reviews, 2016), promotion of ketone production (Kook Hwan Kim et al., 2013), increasing energy consumption (Fisher et al., 2012) and enhancing glycaemic control & glucose uptake in tissues (Berglund et al., 2009). FGF21 binds with the receptor complex comprised of FGF receptor “FGFR1c” and B-Klotho (KLB) (Sally Yu Shi et al., 2018), an obligate co-receptor. FGFR2 & FGFR3, both isoforms of FGFR1c can bind FG21 & KLB, however it is noted that FGFR1 is particularly important for downstream effects of FGF21 expression (Kilkenny and Rocheleau, 2016). The receptor FGFR1 is found throughout mammalian tissues, whereas KLB is instead restricted to a limited number of metabolically active tissues, such as the liver, and adipose tissues (Kurosu et al., 2007). KLB is shown to be a vital component to the metabolic function of FGF21, as shown by the complete elimination of the effect of FGF21 observed in KLB (Ding et al., 2012). Expression of FGF21 is largely limited to the Liver, as such it is referred to as a “hepatokine” (Bourdon et al., 2022). Interest in FGF21 has seen a marked increase in recent years, as it has been shown to function as a potential therapeutic, a biomarker and a target for precision medicine as a potent regulator of our metabolism.

1.2 Metabolic Role:

The hepatokine FGF21 plays a major metabolic role, increasing glucose uptake, alongside inducing insulin sensitivity (Berglund et al., 2009). It has also been referred to as the “starvation” hormone, as it is robustly induced by hypocaloric diets, possibly as a mechanism to aid in the recovery of nutrients and caloric intake post starvation (Zhang et al., 2012).

Ketogenic diets, Low protein diets, High sugar diets and High fat diets are each shown to induce FGF21, with the maximal expression observed in Low protein, high sugar diets (Solon-Biet et al., 2016).

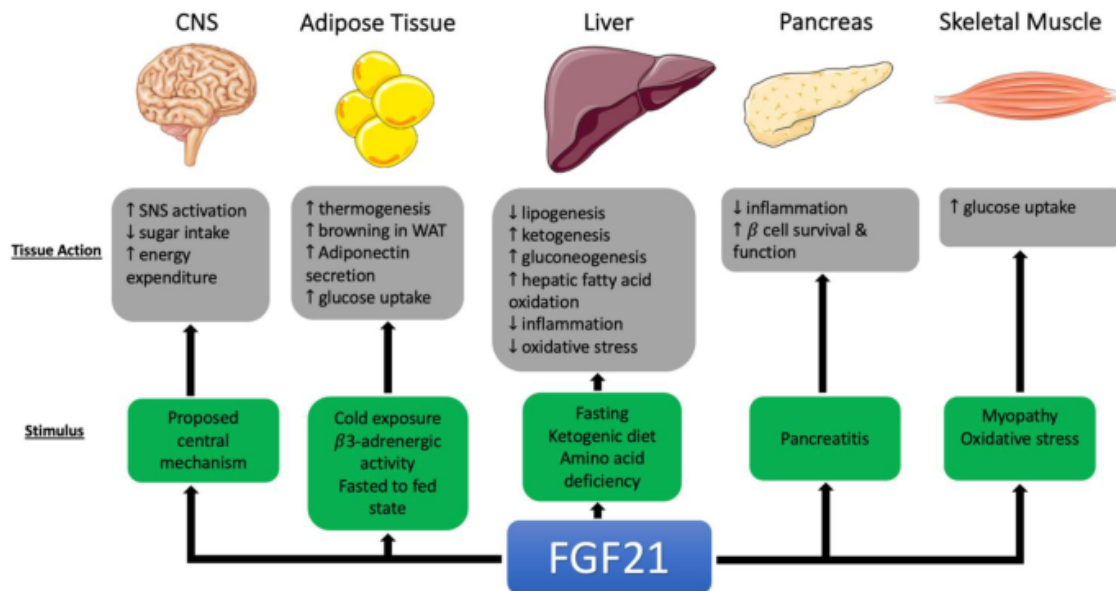


Figure 1: Graphical representation of the multitude of downstream effects of FGF21 expression. Stimuli for expression are listed in green, their respective downstream effects are listed in grey. FGF21 is shown to be induced by a wide variety of factors, both exogenous & endogenous. Moreover, FGF21 also induces a variety of downstream effects in CNS, Adipose Tissues, Liver, Pancreas & skeletal (rough) muscle. (Tucker et al., 2022)

CNS: Central Nervous System

Metabolic disease:

FGF21 is shown to be overexpressed in patients (and animal models) afflicted with metabolic disorders, such as Non-Alcoholic-Fatty-Liver Disease (NAFLD) (He et al., 2017), Alcoholic Fatty Liver disease (AFLD), cardiovascular disease, obesity, amongst others. It is suggested that FGF21 may be used as a potential biomarker for these metabolic disorders, as it is overexpressed in blood serum levels, possibly as patients afflicted with these are insensitive to

FGF21 (Fisher et al., 2010). In particular, Mitochondrial translation disorders may be indicated by increased FGF21 serum levels (Lehtonen et al., 2016). These defective mtDNA translations are the most commonly inherited group of metabolic disorders, arising from the maternal gamete, guaranteeing its proliferation in each offspring (Lehtonen et al., 2016). As a vast group of disorders (>150 genes of interest), a general biomarker such as FGF21 is extremely useful, as it correlates with severity of disease prognosis and other deficiencies within the respiratory chain found in the lumen of the mitochondrion (Lehtonen et al., 2016). Growth Differentiation Factor 15 (GDF15) may also be used in conjunction with FGF21, as a screening tool for these diseases, with the use of FGF21 as an indicator for the severity of each prognosis (Li et al., 2022).

Furthermore, FGF21 is also indicated to play a metabolic role in heart disease and may be used as a biomarker for coronary artery disease (CAD) (Tucker et al., 2022). Lakhani et al noted elevated levels of FGF21 in several disorders, such as CAD, Diabetes Mellitus Type II, Diabetic Nephropathy & Cardiovascular Mortality, all significantly detrimental conditions (Lakhani et al., 2018). FGF21 is associated with several negative health outcomes for both Mice and Human subjects, while not all directly caused by FGF21 (such as being caused by mtDNA translational errors), its overexpression in these significant metabolic disorders warrants caution for its induction through various exogenous stimuli.

1.3 Therapeutic applications of FGF21

FGF21 is also currently studied for its potential use as a therapeutic agent, particularly for diabetes and the ameliorative effect on fatty liver disorders (NAFLD & AFLD) (He et al., 2017). As mentioned previously there is limited KLB expression in the brain, however studies have shown that FGF21 may influence dietary preferences in humans, as in rodent studies

higher levels of FGF21 in nervous tissue were associated with reduced intake of sugars and alcohol, with an increase in protein uptake (Hill et al., 2019).

FGF21 is also noted to have several indirect cardiovascular benefits, through activation of the ERK cellular signalling pathways (Tucker et al., 2022). Tucker et al., 2022 note these effects, as outlined above in Figure 2. Upon FGF21 binding to the KLB/FGFR1c complex on a cardiomyocyte, ERK is phosphorylated, which in turn phosphorylates the MAPK & CREB proteins respectively (Tucker et al., 2022). UCP3 & SOD2 are both upregulated, which in turn reduces Reactive Oxygen Species (ROS), resulting in lowered oxidative stress within the cardiac tissue (Tucker et al., 2022). The AMPK pathway activation reduces apoptosis in the cardiac muscle, reducing cardiac hypertrophy (Tucker et al., 2022). PGC1- α , produced from the ERK/CREB pathway inhibits the NF κ B pathway, which induces inflammatory cytokines, in turn reducing inflammation within the heart (Tucker et al., 2022). These cardioprotective effects may provide some level of protection against the development of heart disease, FGF21's activation of these pathways may prove to have an ameliorative effect on these cardiovascular disorders (Lakhani et al., 2018). The use of exogenous FGF21 in targeted cardiac administration may prove to have clinically beneficial effects.

Furthermore, a study by Pan et al in 2021 demonstrated a “fusion protein”, dubbed “GLP-1-Fc-FGF21”, proved to have potent metabolic effects, greatly aiding in increasing glycaemic control, reduction in weight, and reduction in the presence of liver steatosis in mouse subjects (Pan et al., 2021). Pan et al, also note these beneficial effects are greater than either simple GLP-1 or FGF21 administration, prolonging the drug half-life by recombining these molecules onto the IgG4 Fc region into a dual agonist (Pan et al., 2021).

Evidently, there is significant promise for FGF21 and its use as a therapeutic agent and may be utilised for its ameliorative effects on Diabetes Mellitus Type II, NAFLD, and other such related disorders (Pan et al., 2021).

FGF21, as a “pleiotropic” peptide hormone, promotes a variety of beneficial effects in both human and animal subjects, and shows significant promise for further development and research into its multitude of potential benefits.

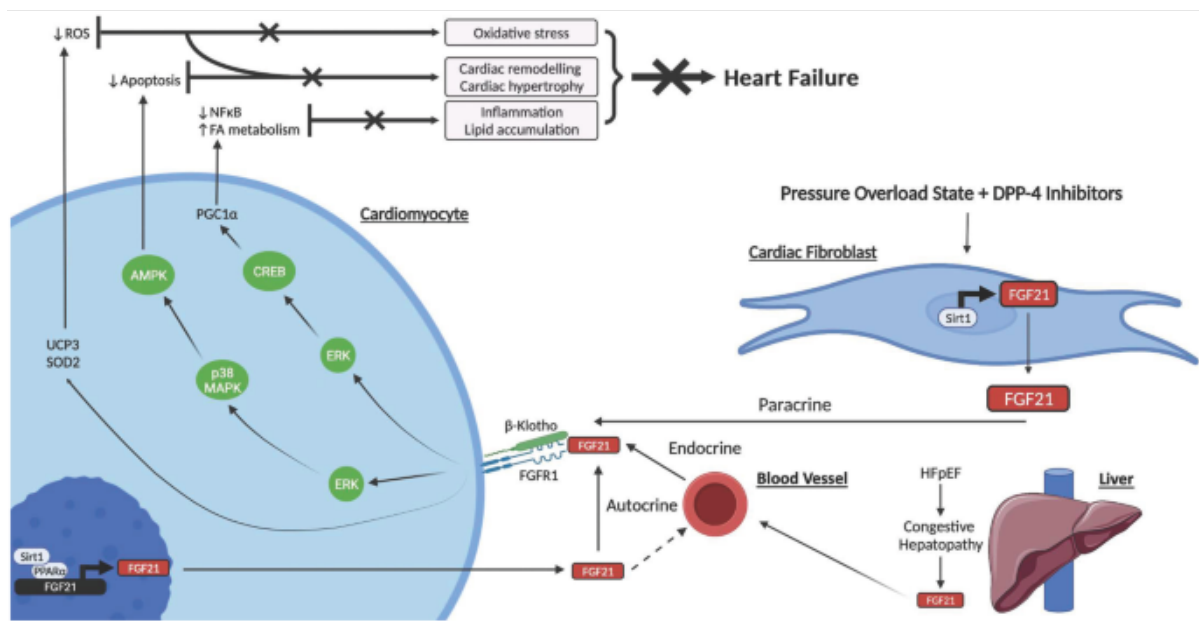


Figure 2: Graphical representation of the cardioprotective downstream effects of FGF21. FGF21 expression triggers three pathways from binding to FGFR1c on the surface of Cardiomyocytes. ERK protein commences two such pathways, which lead to AMPK activation & PGC1- α upregulation respectively. These lead to a decrease in apoptosis, reduction in pro-inflammatory NF κ B & increase in Fatty-Acid metabolism respectively. The third pathway upregulates UCP3/SOD2 which decreases Reactive Oxygen Series. These each contribute to reduced oxidative stress, hypertrophy, remodeling, lipid accumulation & inflammation (Tucker et al., 2022).

ERK: Extracellular-signal regulated kinase, AMPK: adenosine monophosphate-activated protein kinase, PGC1- α : Peroxisome proliferator-activated receptor- γ coactivator, NF κ B: Nuclear factor kappa B, UCP3: Mitochondrial uncoupling protein 3, SOD2: Superoxide dismutase 2.

1.4 Morphological Influence

FGF21 has been shown to be fundamental in the adaptation of mice to chronic cold exposure, as an essential component of “thermogenic recruitment” of White Adipose Tissues (WAT’s) (Cuevas-Ramos et al., 2019). Fisher et al., 2012, demonstrates a severe hindrance of thermogenic recruitment of WATs in FGF21 deficient mice, as FGF21 acts to upregulate UCP-1 and other such thermogenic genes (Fisher et al., 2012).

FGF21 is also noted to have an insulin-sensitising effect, increasing glycaemic control in an adipose tissue dependent manner (Lewis et al., 2020). Studies conducted by Berglund et al., 2009 & BonDurant et al., 2018 demonstrate that FGF21 expression increases glycogen storage and lowered glucagon, shown to function through adipose tissue signalling, independent of adiponectin in BAT & WAT, increasing glucose uptake and reducing lipolysis respectively.

A study by Fisher et al., in 2012, noted the “browning” effect of FGF21 administration to *in vitro* adipose tissue cells harvested from infant mice subjects (Fisher et al., 2012). FGF21 is noted to increase thermogenic gene upregulation, specifically of CIDEA & UCP- (Fisher et al., 2012). Interestingly, specific adipose tissue deposits, such as Inguinal WAT (IWAT) develop the strongest thermogenic gene upregulation when exposed to FGF21 (Fisher et al., 2012). Fisher et al., also note the large increase in “multi-locular” brown adipose cells in the IWAT *in vitro* testing (Fisher et al., 2012).

Furthermore, Epididymal Adipose Tissue (EAT), the liver & pancreas are all noted to be the primary targets of FGF21 secretion (Fisher et al., 2012), however, as discussed previously, it is also suggested that it is secreted in other tissues, not exclusively in the liver, despite the moniker of “hepatokine”. EAT, similar to IWAT, experiences browning when exposed to FGF21 both *in vitro* and *in vivo* (Fisher et al., 2012).

FGF21 induces UCP-1 expression, promoting non-shivering thermogenesis, leading to higher rates of energy expenditure and browning of adipose tissue (Sabrina Azevedo Machado et al.,

2022). In conjunction with this, FGFR1c activation, independent of WAT, promotes noradrenaline & irisin release, which also contribute to UCP1 expression, which promotes thermogenesis in the inner membrane of the mitochondrion, and promotes PGC-1- α receptor upregulation on the cellular membrane of WAT, which contributes to browning of this tissue (Cuevas-Ramos et al., 2019). As a “pleiotropic” hepatokine, FGF21 has a multitude of effects, most notably adipose tissue dependent & independent signalling, which has a range of metabolic effects, including the promotion of browning in WAT’s, improved glycaemic control through WAT expansion (Cuevas-Ramos et al., 2019), and increased energy expenditure in WAT deposits, which may lead to loss in overall weight.

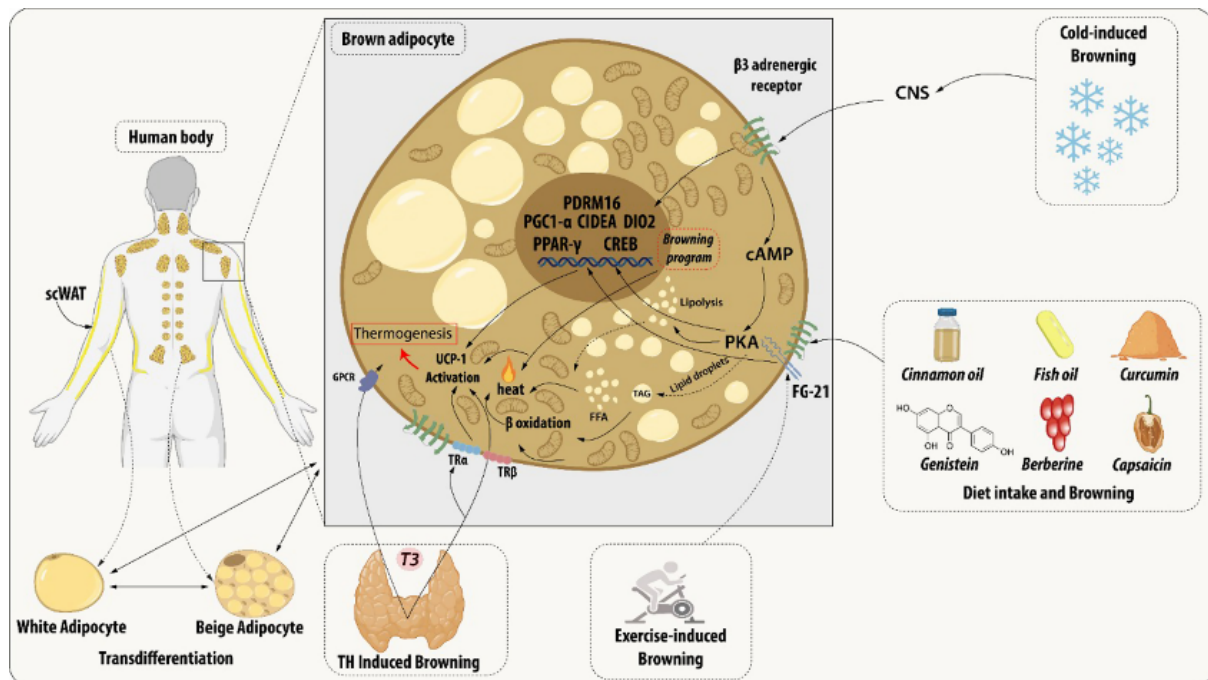


Figure 3: Illustration of metabolic pathways leading to the “browning” of Adipose Tissue. Factors include dietary intake (such as berberine, fish oil etc.) & exercise which induces FGF21, whereas other factors, such as Thyroid Hormone & B-3 adrenergic activity (induced by cold exposure) also contribute. TH browning directly promotes thermogenesis, whereas FGF21 & B-3 adrenergic stimuli also promote several genes which promote AT browning. (Sabrina Azevedo Machado et al., 2022)

1.5 Diet & FGF21:

FGF21 is differentially expressed in mammalian systems dependent on the constituents of a diet. An important factor is the proportion of each macronutrient present: Carbohydrates, Fats & Protein (Solon-Biet et al., 2016). In particular, low protein and high carbohydrate are noted to upregulate FGF21 (Solon-Biet et al., 2016). Described as the “starvation hormone” FGF21 is also highly upregulated in hypocaloric diets (Luisa et al., 2013).

One such study, conducted by Solon-Biet et al., in 2016, denotes that a low protein intake coupled with high carbohydrate intake promotes the highest level of FGF21 expression & circulation (Solon-Biet et al., 2016). For individual macronutrient analysis, FGF21 plasma levels negatively correlate with protein intake ($R = -0.58$ & $P < 0.01$) whereas it is positively correlated with carbohydrate intake ($R = 0.488$ & $P < 0.001$) (Solon-Biet et al., 2016). This is noted to function in conjunction with caloric intake (Solon-Biet et al., 2016), where mice consuming $< 5\text{kJ/day}$ expressed higher FGF21 levels both in serum & RNA expression ($P < 0.001$ & $P < 0.001$ respectively) (Solon-Biet et al., 2016). Solon-Biet et al. also note that FGF21 expression is upregulated in overfeeding, obesity & insulin resistance (Solon-Biet et al., 2016), however the full metabolic effect does change dependent on each factor present which induces FGF21 expression (Solon-Biet et al., 2016). Solon-Biet et al. note that the primary reason for overexpression of FGF21 (both RNA & serum level) in this study is protein and amino acid restriction (Solon-Biet et al., 2016).

FGF21 is primarily induced through High Fat, High Carbohydrate & Low protein diets, evidenced across a variety of studies, where High Carbohydrate & Low Protein combination diets are shown to maximally express FGF21 both in serum & in RNA (Solon-Biet et al., 2016).

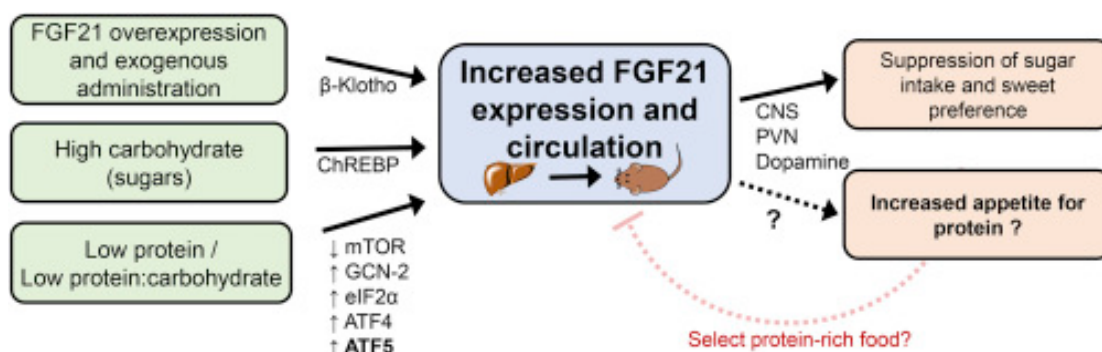


Figure 4: Illustration of the factors which influence an increase in joint FGF21 expression & circulation. Dietary factors include low protein & high carbohydrate content which influence mTOR, GCN-2, eIF2a, ATF4, ATF5 & ChREBP levels respectively. Alternative factors include FGF21 overexpression and exogenous FGF21 delivery, which influence KLB expression. FGF21 upregulation is suggested here to influence the suppression of sugar intake & increasing protein appetite (Solon-Biet et al., 2016).

mTOR: mammalian target of rapamycin, GCN-2: general control nonderepressible 2, eIF2a: Eukaryotic translation initiation factor 2A, ATF4: Activating transcription factor 4, ATF5: Activating transcription factor 5, ChREBP: Carbohydrate response element binding protein.

FGF21 influence on diet.

Not only is diet an exemplary influence on FGF21 expression both in serum and tissue RNA levels, FGF21 is also shown to influence dietary choice, influencing the level of different macronutrient intake in mouse models (Hill et al., 2019). Hill et al. demonstrate the effect of FGF21 induction through protein restriction, and the subsequent effect of this restriction, showing a link between this and the food preference of the mice present in their trial (Hill et al., 2019). The mice, when presented with a protein restricted diet experienced robust FGF21 expression, which as mentioned previously induces a variety of metabolic effects, such as an

increase in thermogenesis, increase in energy expenditure, and an increase in glycaemic control (Hill et al., 2019). When given a chronically low protein diet, these mice exhibit these adaptations to the food, and increase their overall intake (Hill et al., 2019). Interestingly, when given an alternate protein source, external from this diet, the mice will ingest the available protein, without ingesting larger amounts of food (Hill et al., 2019). This demonstrates a clear preference for higher protein food in lower amounts, however these mice are capable of adapting to chronic protein malnutrition (Hill et al., 2019).

Specifically, KLB Knockout (Klb^{Camk2a}) & KLB control ($Klb^{lox/lox}$) mice strains were utilized here, where a low-protein diet increased casein and reduced maltodextrin intake in $Klb^{lox/lox}$ control mice ($P < 0.05$) and induced a strong preference for casein intake ($P < 0.05$) (Hill et al., 2019). This effect however is contrasted with Klb^{Camk2a} mice, which had no statistically significant differences between their preferences, regardless of diet (Hill et al., 2019). As KLB is an essential co-factor for the binding of FGF21 with FGFR1c, this is demonstrative of a link between FGF21 activity and macronutrient preference in these mice (Hill et al., 2019). This link is further supported by the comparison of this preference between FGF-KO mice & Wild-type mice, where after a period of a low protein diet, only the “wild” mice strongly prefer a higher casein diet after chronic protein malnutrition ($P < 0.05$) (Hill et al., 2019). As is well established, FGF21 is a vital metabolic hormone, which not only influenced by dietary intake, but is also, conversely, an influence on macronutrient preference (Hill et al., 2019). Acting as a regulatory force in malnourished rodent models, FGF21 is both vital to and directly influential to a variety of metabolic systems, as macronutrient preference will ultimately determine FGF21 expression, which in turn influences its overall metabolic effect (Hill et al., 2019).

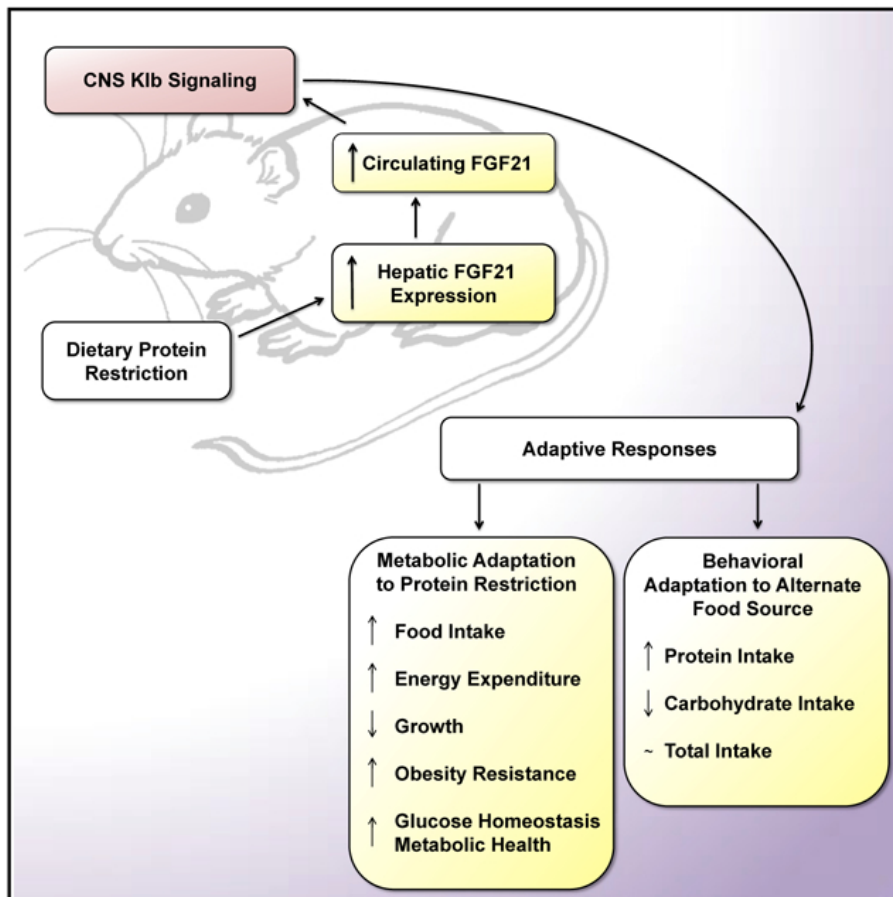


Figure 5: Graphical representation of the effect of FGF21 expression induced by protein restriction. PR induces FGF21 expression, which interacts with KLB in the Central Nervous System. This has two forms of adaptive response: Metabolic Adaptation & Behavioral Adaptation, respectively. Metabolically, mice adapt by increasing Food Intake, Energy Expenditure, Insulin sensitivity and reduce overall growth. Behaviorally, Mice increase protein intake and reduce carbohydrate, maintaining standard overall caloric intake. (Hill et al., 2019)

1.6 Mouse Tissues:

Epididymal Fat

Epididymal Adipose Tissue (EAT) is a sub-type of White Adipose Tissue (WAT), forms the majority of adipose tissue in mammals, used as reserve stores of energy, which is then metabolized in times of calorie deficit. A study conducted by Chu et al. in 2010 demonstrated that EAT is essential for spermatogenesis but has no impact on copulation or testosterone in male Syrian hamsters (Chu et al., 2010). Inguinal Adipose Tissue (IAT; a non-gonadal White Adipose Tissue) lipectomy, in contrast to EAT lipectomy, had no effect on spermatogenesis (Chu et al., 2010). EAT lipectomy also reduced the size of the seminiferous tubules, with a marked decrease in Sertoli cells & spermatogonia (Chu et al., 2010). This lipectomy also doubled the serum levels of Follicle Stimulating Hormone (FSH), in contrast to the lack of effect on Luteinizing Hormone (LH) & Testosterone (Chu et al., 2010). Evidently, EAT is important for the full sexual function of the male gonads, aiding in the growth and proliferation of the spermatogonia and growth of the seminiferous tubules through an unknown metabolic factor (Chu et al., 2010).

Furthermore, as established previously, EAT is a highly active metabolic tissue (Dai et al., 2022). Infiltration of adipose tissue with macrophages (Adipose Tissue Macrophages; ATM's) is a widely noted pathological effect of obesity, of which the ATM's secrete a variety of pro-inflammatory cytokines, such as Tumour Necrosis Factor- α (TNF- α) & Interleukin 1-B (IL1-B). Dai et al., 2022, note the secretion of osteopontin from EAT when mice are fed high fat diets (HFD) (Dai et al., 2022). Osteopontin (OPN) has a known role in the recruitment of ATMs into adipose tissues and is robustly produced by EAT when mice are subjected to HFD's (~28x) (Dai et al., 2022). In conjunction with ATM recruitment, OPN also regulates the mineralization of bone matrices and the attachment of bone cells, both important roles in proper skeletal function (Dai et al., 2022). OPN is an inflammatory cytokine, which promotes the chemotaxis

and motility of macrophages, promoting their infiltration into adipose tissues, forming ATM's (Takashi Nomiyama et al., 2007). Chronic HFD feeding in mice was noted to induce OPN, which in turn induced ATM infiltration, amplifying the pathologies of obesity, including the promotion of chronic low-grade inflammation in these tissues (Takashi Nomiyama et al., 2007). Interestingly, OPN deficient murine subjects demonstrated increased glycaemic control, contrasted by the relative insulin resistance in standard obese mice, in conjunction with increased ATM infiltration (Takashi Nomiyama et al., 2007). Notably OPN deficient mice displayed a reduction in pro-inflammatory cytokine markers, such as IL-6, MCP-1, and PAI-1 (Takashi Nomiyama et al., 2007), which is further supported by the relative reduction in ATM infiltration (Takashi Nomiyama et al., 2007).

Epididymal Adipose Tissue is a vital metabolically active tissue, contributing to the maintenance of bone matrices (Dai et al., 2022), the promotion of low-grade obesity induced inflammation (Takashi Nomiyama et al., 2007) and the promotion of spermatogonia, seminiferous tubules and spermatogenesis, contributing to the full function of male gonads (Chu et al., 2010). EAT is also noted to be a primary target of FGF21 function (Fisher et al., 2012).

EAT, as such an active tissue, may be affected in a variety of ways by FGF21, disregarding the previously noted effects on adipose tissues, such as increased proliferation, increased glucose uptake & increases in energy expenditure and browning (Fisher et al., 2012). However, there may be a link between FGF21, its action on EAT & spermatozoa (Bourdon et al., 2022). While this study has been conducted in human subjects and is independent of EAT, the noted effect of EAT and its contribution to male gonadal function may suggest a potential link between each of these elements (Bourdon et al., 2022).

Liver

Liver steatosis is defined as the excessive fat accumulation in the hepatocytes of the liver, forming >5% of total liver weight (Echeverría et al., 2019). This encompasses two distinct disorders; Non-Alcoholic Fatty Liver Disease (NAFLD) & Alcoholic Fatty Liver Disease, NAFLD is the most common liver disorder across the world, particularly for higher fat and processed diets, such as in “western” countries (Echeverría et al., 2019).

Steatosis of the liver is the culmination of metabolic disorder and is associated with conditions such as obesity, decreased glycaemic control and oxidative stress (Echeverría et al., 2019). Similarly, to EAT, pro-inflammatory cytokines are also expressed here, in coalition with these conditions (Echeverría et al., 2019). Echeverría et al. note that NAFLD is induced by chronic High Fat Diets, with a noted decrease in energy metabolism (Echeverría et al., 2019). HFD's were seen to robustly induce liver steatosis, promoting a 66% decrease in n-3 long chain polyunsaturated fatty acids (N-3 PUFAs), alongside a 100% increase in n-6/n-3 LCPUFA ratio when compared to the control diet group ($P < 0.05$) (Echeverría et al., 2019). Both N-3 PUFA depletion & increases in the N-6/N-3 PUFA ratio are strongly associated with liver steatosis (Echeverría et al., 2019).

Liver steatosis is however, noted to be ameliorated by the overexpression of FGF21 (Yano et al., 2022). Mice treated with hydronamic FGF21 at 6-week intervals demonstrated both higher levels of FGF21 in circulation, in conjunction with decreased markers of liver steatosis (Yano et al., 2022). Furthermore, FGF21 promoted lipolysis and helped prevent lipogenesis via the inhibition of sterol regulatory element-binding proteins (Yano et al., 2022), which are vital to the generation of new adipose tissue. In addition to these beneficial effects, FGF21 treatment also inhibited glucose-6-phosphate and phosphoenolpyruvate carboxylase, which are both required for gluconeogenesis (Yano et al., 2022), therefore promoting glycaemic control by reducing the total amount of circulating blood sugar (Yano et al., 2022). Evidently, FGF21 acts

in a beneficial manner to reduce NAFLD alongside other obesity related conditions and can ameliorate the worst pathologies associated with chronic HFD feeding (Yano et al., 2022). Echeverría et al., 2019, also demonstrated that eicosapentaenoic acid (EPA), a form of N-3 PUFA ameliorated the worst effects of HFD induced NAFLD, when it was added to the rodent HFD's (Echeverría et al., 2019). This may be due to an induction of FGF21 expression, as a study conducted by Escoté et al. in 2018 demonstrated that EPA supplementation into the diets of human subjects induced circulating FGF21 expression (Escoté et al., 2018). EPA supplementation was also observed to influence the N-6/N-3 PUFA ratio, decreasing N-6 PUFA's while increasing N-3 PUFA's (Escoté et al., 2018). This is also contrasted with the effect of HFD's in mice, where the opposite effect is observed (Echeverría et al., 2019). As these are markers of liver steatosis, and FGF21 is observed to ameliorate and reverse symptoms of liver steatosis (Echeverría et al., 2019), this may be a link between EPA supplementation, FGF21 expression, and reduction in liver steatosis markers. While this would require further study and inquiry, it is well established that FGF21 overexpression/treatment led to the improvement of liver steatosis, and other related metabolic conditions (Yano et al., 2022).

1.7 Allulose

Allulose is a rare monosaccharide, found in several different sources, such as small amounts in brown, unrefined sugar & dried fruit (Ahmed et al., 2021). Allulose is an epimer of Fructose (Ahmed et al., 2021), where the hydroxyl group connected to the C-3 molecule on the cyclic structure of Fructose is flipped to the opposite side (Ahmed et al., 2021). Despite near identical molecular structure, this single molecular flip creates a vast difference in overall metabolic effect when it is consumed, constituting a mere 10% of the overall calories of Fructose (Ahmed et al., 2021). The majority of consumed Allulose (~70%) is absorbed in the small intestine, where it is robustly secreted through urination (Ahmed et al., 2021). The remaining ~30% is

transported to the colon, where it remains unfermented and is also fully excreted within ~48hrs (Ahmed et al., 2021). Allulose is transported through the gut in an identical fashion to Fructose, where GLUT5 (Fructose transporter type 5) facilitates enterocyte transmembrane (Ahmed et al., 2021). With such a low-calorie density and a relatively high level of sweetness (~70% sweetness of Fructose) (Ahmed et al., 2021), Allulose may be a beneficial alternative to “traditional” sweeteners, such as Fructose.

Not only does Allulose reduce the sheer number of calories consumed, it is also linked with several beneficial effects on mammalian systems (Lee et al., 2020). A recent study conducted by Lee et al in 2020 observed the effect of Allulose supplementation on genetically obese, C57BL/KsJ-db/db mice (Lee et al., 2020). Contrasted with a “Normal diet” control and a 5% erythritol supplemented diet, Allulose significantly reduced the signs of obesity (Lee et al., 2020). Most notably, subcutaneous WAT (white adipose tissue), epididymal WAT, mesenteric WAT and total WAT were all significantly decreased (Lee et al., 2020) when compared to both normal and 5% erythritol diets. Furthermore, liver steatosis was also decreased, in conjunction with the suppression of several key genes which contribute to hepatic lipid accumulation, such as FAS (fatty acid synthase) & G6PD (glucose-6-phosphate dehydrogenase) (Lee et al., 2020). In addition to this, Allulose also reduced serum glucose & glucagon content, while preserving Islet of Langerhans, suggesting a boon to insulin sensitivity & the prevention of diabetes mellitus II (Lee et al., 2020).

Allulose demonstrates significant promise not only as an alternative sugar which would robustly reduce overall caloric intake, but also with the promotion of highly beneficial downstream metabolic effects. Allulose may be an influence on the expression of FGF21, which would provide several noted metabolic effects.

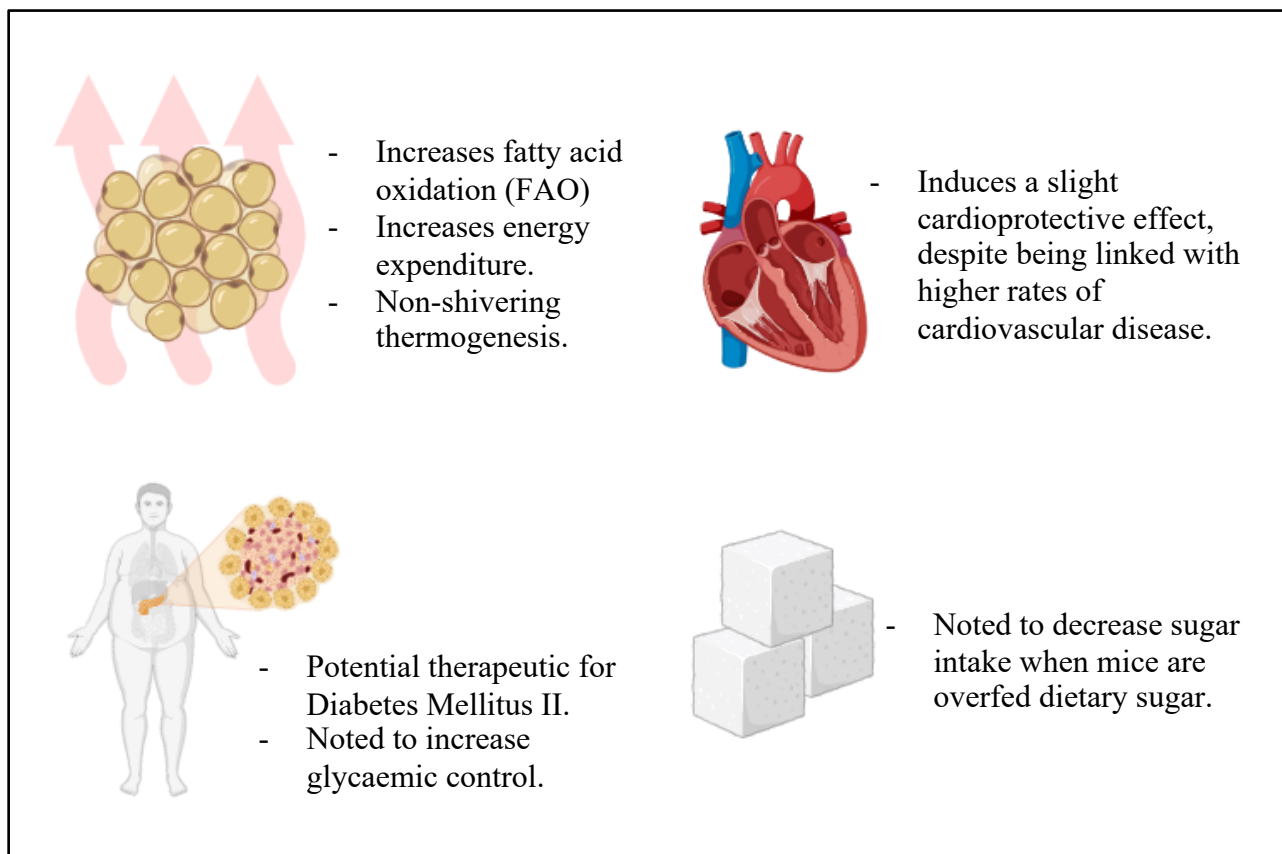


Figure 6: Graphical summary of several effects of FGF21 secretion. This encompasses the metabolic effects and the therapeutic & dietary influences. FGF21 is a multifaceted hormone, but its primary downstream effects encompass increased glycaemic control & increases to both thermogenesis and browning of AT's (Created with Biorender.com).

1.7.1 Fructose

Sucrose is a naturally occurring plant sugar, formed through photosynthesis within a multitude of plants (Herman et al., 2021). Sucrose is a disaccharide with two disparate subunits: Glucose, the main sugar used by mammalian metabolisms, and Fructose (Herman et al., 2021). Fructose is converted to Glucose and also converted to the polymeric glycogen where necessary by mammals, where it is rapidly absorbed across the gastrointestinal (GI) tract (Herman et al., 2021). While Fructose may be beneficial to glucose storage and the regulation of blood sugars, specifically for the replenishment of these, it is unfortunately linked with several negative

effects, particularly for chronic over-indulgence (Messier et al., 2007). Several animal studies have been indicative of high fructose intakes inducing hyperinsulinemia, obesity and hypertension (Messier et al., 2007). Messier et al. describe deleterious effects induced by High Fat (HFD) content, whereas the addition of Fructose did not impact the overall deleterious effects of HFD, such as the increased weight gain (Messier et al., 2007), however this does not detract from the fact that high Fructose content is noted to robustly induce weight gain (Messier et al., 2007), independent of HFD, which introduces several negative health implications.

Curiously, Fructose is a noted regulator of FGF21 secretion (Fisher et al., 2017), in particular inducing robust hepatic mRNA upregulation & circulating levels (Fisher et al., 2017), whereas the increase in WAT mRNA is decreased when compared to Dextrose supplementation, although still elevated overall (Fisher et al., 2017). Fisher et al also further demonstrate that Carbohydrate response element binding protein (CHREBP) knockout mice experience decreased FGF21 induction, suggesting FGF21 is induced at least in part by CHREBP (Fisher et al., 2017). Evidently, fructose has a profound effect both on the weight of mice, but also the sugar metabolism, particularly FGF21 expression.

As such, a Fructose-supplemented HFD was adopted as an intervention to contrast against Allulose supplementation. As Fructose is noted to induce several negative effects, both in combination with and independent of HFD, it may act as a strong contrast if there is any ameliorative effect present from Allulose supplementation.

1.8 Summary of introduction & Hypothesis

In conclusion, FGF21 has a multitude of metabolic effects, ranging from several negative correlations such as heart disease and different pathologies of obesity, where it is often overexpressed. In contrast to this, localized FGF21 expression/administration is linked with many positive outcomes, such as the amelioration of NAFLD, reduction in weight, and the

improvement of glycaemic control. Allulose, as a rare and very promising subject for research, demonstrates a variety of benefits, both in terms of its reduced caloric density, to its downstream effects on mammalian systems. If Allulose alters the expression of FGF21, there is significant promise for Allulose supplementation, independent of the previously reported benefits, as FGF21 is noted for its multitude of positive effects. This has led to the formation of the hypothesis:

“Allulose supplementation alters the expression of FGF21, both in adipocyte & serum expression, leading to the promotion of several beneficial metabolic effects.”

1.9 Objectives

This study aims to assess the effect of Allulose sugar supplementation in a High Fat Diet, and to analyse its effect on FGF21 in tissue & serum expression.

1.9.1 Specific Objectives

- (i)*** To analyse the RNA expression of FGF21 in adipose tissue samples (EAT), through the use of Quantitative real-time PCR.
- (ii)*** To analyse the serum expression of FGF21 protein in each group utilizing statistical analysis, from data generated previously utilizing ELISA assays.
- (iii)*** To assess the overall role & effect of FGF21 across each group.
- (iv)*** To assess the overall effect of Allulose supplementation, its relationship to FGF21 expression & total metabolic effect when supplementing a High Fat Diet.

2.0 Materials & Methods:

2.1 Animal models

80 C57/BL6 mice were utilized in a study conducted in 2019 by Dr Catherine Staunton & Dr Fabiana Hoffmann Sarda in Teagasc, Moorepark, Co. Cork.

These mice were subjected to 14 weeks of one of 4 diets, fed *ad libitum*: Lean Diet (LD) (n=10), High Fat Diet + Water (HFDw) (n=10), High Fat Diet + Allulose (HFDa)(n=12) & High Fat Diet + Fructose (n=12) (HFDf). Mice were subjected to serum analysis provided from their blood and were euthanized by cervical dislocation after the commencement of the study. Tissues were separated and stored in -80 ° Celsius cold storage until the undertaking of this study. 10x LDw, 9x HFDw, 12x HFDf & 12x HFDa mice were selected for statistical analysis & tissue processing. One mouse from group HFDw was excluded due to inadequate management during the OGTT test.

The experimental design and methods used to obtain the data set used in this thesis are in the Appendix.

2.2 RNA Extraction materials & methods

RNA extraction was performed on 36 EAT samples from all four groups: 10x LD, 6x HFDw, 10x HFDf & 10x HFDa. Of 9 HFDw mice, 3 samples were irretrievable.

Samples were placed in 2mL Lysing Matrix D tubes [MP Biomedicals, Santa Ana, CA, USA], suspended in a 1% solution of β -mercaptoethanol [Scientific Laboratory Solutions, Nottingham, UK] in buffer RLT from an RNEasy Minikit [Qiagen; Citylabs 2.0, Manchester, UK]. Samples were then placed in a Fastprep 24 5g bead beater & lysis instrument [MP Biomedicals, Santa Ana, CA, USA] and subjected to a pre-set 40 second cycle. Lysate was transferred to a room temperature centrifuge and spun at 10,000 RCF for 180 seconds. 600 μ l of lysate was transferred to spin columns from a Qiagen RNEasy kit and spun for 30 seconds

at 10,000 RCF. Samples were washed with 600 μ l of buffer RW1 and re-spun, before DNase 1 [Qiagen; Citylabs 2.0, Manchester, UK] treatment for 15 minutes, after which they were washed and subsequently re-spun each time with RW1 and twice with Buffer RPE. Spin columns were placed into fresh 2mL collection tubes before being washed with 50 μ l H₂O and spun at 10,000 RCF for 1 minute. RNA extracts were divided into aliquots and stored at -80 ° Celsius. RNA concentrations were analysed using a BioTek Take3 Nanodrop plate [Agilent, Santa Clara, CA, USA].

2.3 Complementary DNA library generation

RNA extract concentrations were used to calculate the required amounts for the preparation of “Reverse transcription master mix” utilizing the Quantitect Reverse transcription kit [Qiagen; Citylabs 2.0, Manchester, UK]. 1 μ l of RT Primer Mix, 1 μ l of Reverse Transcriptase and 4 μ l of Quantiscript RT Buffer 5X were added, a volume of RNA extract was added (on average 15 ng/ μ L), the final amount of water was added to add up to 20 μ l reaction volume. The RNA concentration was standardized to the lowest concentration of RNA present amongst the set of samples, so as to equalize the concentration between reactions.

Once prepared, samples were incubated at 42 °C for 15 minutes before heating to 95 °C for 3 minutes to denature the reverse transcriptase enzyme. Samples were then analysed using a BioTek Take3 Nanodrop plate [Agilent, Santa Clara, CA, USA].

2.4 Quantitative Polymerase Chain Reaction

Peptidylprolyl isomerase A (PPIA) was chosen as another gene for analysis as it is a particularly stable “housekeeping gene” (Fan et al., 2020). PPIA expression is noted to be unaffected by High Fat macronutrient stress when applied to rodent models (Fan et al., 2020), in contrast with more commonly used housekeeping genes, such as GAPDH (Fan et al., 2020)

. “Housekeeping genes” are used for reference against the gene of interest, and may indicate sample degradation, where CT scores are measured in relative values in lieu of an absolute concentration of material.

FGF21 qPCR primers [Integrated DNA Technologies Inc., Coralville, IA, USA] were custom designed using IDT DNA’s website design tool:

FGF21 Forward Primer: 5’CTACACAGATGACGACCAAGAC 3’

FGF21 Reverse Primer: 5’ CTTTGAGCTCCAGGAGACTTTC 3’

PPIA qPCR primers were pre-designed on IDT DNA’s website:

PPIA Forward Primer: 5’ CAAACACAAACGGTTCCCAG 3’

PPIA Reverse Primer: 5’ TTCACCTTCCCAAAGACCAC3’

Primers were prepared into aliquots of 0.5mM concentration, diluting 20x using purified water.

Primer master mixes were prepared according to the following measures: 3 µl H₂O, 2 µl primer mix (inclusive of both forward and reverse primer), 10 µl SYBR Green master mix [Thermo-fisher Scientific, Dublin, Ireland]. 10% additional mix was added for each to account for pipette error, calculations are multiplied by each well used for each qPCR run.

MicroAmp Fast-8 Tube strips [Thermo-fisher Scientific, Dublin, Ireland] were filled with samples and sealed with MicroAmp Optical 8-cap Strips [Thermo-fisher Scientific, Dublin, Ireland].

Strips were loaded into StepOnePlus qPCR system [Thermo-fisher Scientific, Dublin, Ireland] and ran at 95 °C for 30s (40 cycles) before 5s at 95 °C, 15s at 60 °C for FGF21, (50 °C for PPIA) and 10s at 72 °C. Melting curve data was also collected as part of each individual run.

2.5 Histological Analysis of Liver

Mice liver samples were fixed in 10% formalin acetate (phosphate buffered) at 4°C overnight, before embedding in sections of paraffin wax (Cui et al., 2017). Liver sections of 5 µm in width

were cut using a Tissue-Tek Cryo3 Flex Cryostat, a freezing microtome which allows for samples to be cut while under freezing conditions. This work was conducted by Dr Joanna Allardyce on our behalf. Two slides from each group (from the same source) were chosen and stained with Oil Red O lipid stain (ORO). This work was conducted together with Ms. Cathy Cullinan & Ms. Megan O' Sullivan McCarthy, Final Year Project students.

ORO solution [Merck, Darmstadt, Germany] was diluted to a 3:2 ratio with double distilled water and filtered using a Whatman filter pad in a plastic funnel. Slides were fixed in formaldehyde vapours from 10% Formalin solution by inverting them and suspending them over a sample of Formalin, which is encased with ice to reduce temperatures to approximately 4C (Cui et al., 2017). Samples were fixed in these vapours for a period of 5 minutes (Cui et al., 2017). Samples were then dipped in 60% isopropanol (2-propanol) solution for 3 seconds, removed, and dipped for a further 3 seconds each (Cui et al., 2017). ORO 3:2 solution was applied to each sample on the slides, where they were incubated for 15 minutes at room temperature while covered, to prevent light exposure from the surrounding environment (Cui et al., 2017). Slides were removed from incubation before being placed in 60% isopropanol for 3 seconds, before removal and further rinsing for 3 seconds in this solution (Cui et al., 2017). Samples were rinsed with double distilled water twice, before removal of excess water around the samples by blotting (Cui et al., 2017).

10% Modified Meyers hematoxylin solution was then prepared, before placing samples in this solution for 60 seconds each (Cui et al., 2017). Samples were then rinsed with tap water for 10 minutes each, before placement in distilled water until cover slips were to be placed on each slide (Cui et al., 2017). Samples were dried of excess fluid and analyzed using an EVOS M5000 Electronic Microscope.

Oil Red O was selected for this staining process as it is a non-polar compound which binds exclusively to lipids within the cytoplasm of the cell. The primary form of lipid storage, the

triglyceride, is non-polar, and as such ORO is exclusive for its affinity to TG's. This allows for the highlighting of lipid deposits across a selection of tissues.

2.6 Statistical Analysis

In the 2019 study, a variety of metrics were measured from each of the mice. These were recorded in a raw-data spreadsheet, which was shared for the purpose of this 2023 study.

Metrics chosen for the purpose of this study were: Weight gain (weeks 11 & 14), Baseline glucose, OGTT results, baseline insulin, Triglyceride amount in liver, Spleen, Liver & pancreas weights, EAT, SAT, Brown, Retro & Mesenteric AT's, Total Adipose Tissue, Leptin, Ghrelin, GLP-1 & FGF21 measurements.

Following RNA extraction, cDNA generation & subsequent qPCR, new metrics were generated from CT scores; Δ CT, FGF21 average CT and PPIA average CT.

Δ CT for each EAT sample analysed was calculated by subtracting the PPIA CT average score from the FGF21 CT average score for each sample (Livak and Schmittgen, 2001).

$\Delta\Delta$ CT was calculated by subtracting the samples (based on diet) from its relative control average Delta (Δ) CT (Livak and Schmittgen, 2001):

HFDa Δ CT – HFDw Average Δ CT
HFDf Δ CT – HFDw Average Δ CT
HFDw Δ CT – LDw Average Δ CT
LDw Δ CT – LDw Average Δ CT

LDw is treated as the control for both HFDw & LDw, while HFDw is treated as the control for HFDa & HFDf, as for the most accurate results, only one degree of change is present between the relative controls and their experimental counterparts.

The $2^{-\Delta\Delta Ct}$ formula was utilized to calculate fold change in respect to each control (Livak and Schmittgen, 2001). This was calculated in Excel [Office; Microsoft] by calculating 2 to the

power of the negative of the values generated by this above calculation. This was followed by the calculation of fold change between groups using the formula:

$\text{Log}(\text{Experimental}/\text{Control} \{2\})$.

The previous data set and all data generated in this study were tested for normality (Shapiro-Wilk) and homogeneity (Levene test) and analysed with parametric or non-parametric tests accordingly. Detailed results are listed below in Appendix table 1.

The Shapiro-Wilk Test is used for each normally distributed set, where Mann-Whitney U is used for non-normally distributed data.

If PPIA CT scores were particularly low or fluctuating, this would be indicative of improperly prepared samples, as PPIA RNA upregulation should stay at similar levels regardless of the diet chosen. As the changes between these scores are shown to be statistically insignificant, it is assumed that generally the samples were of similar quality when subjected to qPCR analysis. A breakdown of specific CT scores, their calculation, and specific statistic tests are listed in Appendices, alongside correlations of note generated with Pearson correlation statistical tests.

2.7 Statistical Imputation

All result groups were subjected to statistical imputation, as “extreme outliers” (more than 3 SD’s away from the median value) present in datasets interfere greatly with overall analysis. Extreme outliers were replaced with (A) the median value of the diet group, or (B) the mean value of the diet group, based on whether the metrics were non-parametric or parametrically distributed respectively.

While it is noted that this does lead to statistical bias, it does not largely change the mean or median values, but instead reduces the overall spread of data. While this is important, it must be noted that the most valuable metrics present remain largely unchanged, regardless of style or amount of imputation present in the final processed data. Specifically, Mice 5, 8, 10, 12, 32,

34, 36, 39, 57, 75 & 79 were imputed for several metrics, as all were listed under “extreme outliers” ($3 < SD$'s away from median values). Upon imputation of these values, mice 4, 6, 33, 34, 76 & 78 were imputed for datapoints also.

This of course is due to bias with the “tightening” of the numeric distribution, leading to a reduced standard deviation for each group, however upon imputation of these values, no values were returned as “extreme outliers” and so this was considered a necessary alteration to the dataset. It must also be noted that this imputation was largely conducted in datasets considered of lesser importance, such as Liver weight, Spleen weight & OGTT metrics.

However, Mice 4, 5, 6, 36 & 78 were imputed for their respective $2^{-\Delta\Delta C_t}$ metrics, which must also be considered in the final analysis of the data present, while again this may lead to somewhat of a bias, it is far more representative of the groups as a whole, as many of these outlier datapoints may have been made due to clerical or systematic experimental error.

Raw data was initially processed using Excel [Microsoft], before processing and transferal to SPSS statistical software [IBM]. This data was treated as two separate categories: Parametrically and Non-Parametrically distributed data. Statistical tests encompassing both sets were conducted when tests did not assume normality, however it is restrictive when considering the whole scope of statistical analysis. As mentioned prior, two separate controls are present in this experiment: HFDw and LDw diets. HFDw serves as a “positive control” for the High Fat experimental diets, specifically HFDA & HFDf, as only one factor is changed between these diets. LDw serves as a “negative control” for HFDw as again only one factor changes between these. For the purpose of the statistical analysis, LDw will only be compared directly to HFDw, whereas HFDw will be compared both to LDw but also HFDf & HFDA respectively.

This is conducted in this manner in an attempt to best clarify the factors influencing the relevant data metrics, as isolation of each factor will prove more difficult without separation of the “negative control” from the experimental diets, as all factors present are completely in contrast. Furthermore, four mice (32, 33, 34 & 41) had their qPCR metrics imputed, as they were not found in storage with the other number of mice samples, however their remaining metrics (such as weight and blood analysis) remain intact from the initial dataset. Furthermore, mice 1, 42, 79, 9, 53, 54 & 56 were imputed for qPCR metrics, due to either unusable data or failure to generate sufficient cDNA for analysis.

2.8: Dietary information & mice interventions

44 C57BL/6J 5-week-old male mice were purchased commercially (Envigo; UK) and were housed 4 or 2 per cage on a 12 hr light/dark cycle. The mice had ad libitum access to food and water throughout the study unless otherwise stated. During the initial 2 weeks of the acclimatization period, mice were provided with a diet containing a 10% low-fat diet (Lean; #D12450B; Research Diets, USA; % by values of energy). Subsequently, weight-matched mice received a high-fat diet (Research Diets, USA (3) 45% fat (HFD-CAS; #D12451) and drinking water, or Fructose drinking solution (4%) and allulose drinking solution (4%). These concentrations were calculated based on previous studies (and maximum daily intake (Han et al, 2018), adjusted to mice body weight. Detailed dietary intervention & caloric information are available in Appendix on page 93 & 95.

3.0 Results:

3.1 ELISA metrics & OGTT results

Table 1: Summary of biochemical analysis

	DIETS							
	LD		HFDw		HFDf		HFDa	
	Mean	ST. Dev	Mean	ST. Dev	Mean	ST. Dev	Mean	ST. Dev
Triglycerides (mg/g liver)	4.95	2.80	6.54	2.37	6.81	5.31	4.64	1.49
Baseline insulin (ng/mL)	0.373	0.17	1.20	0.47	0.90	0.54	0.47	0.23
Baseline glucose (mmol)	5.44	0.70	7.49	1.24	6.90	1.07	6.41	1.11
OGTT 15 mins (mmol)	13.61	1.59	19.47	5.18	17.80	2.20	17.51	2.26
OGTT 30 mins (mmol)	12.41	1.55	15.63	1.58	15.21	1.62	15.38	2.69
OGTT 60 mins (mmol)	10.17	1.13	13.02	1.54	12.30	0.77	12.76	1.79
OGTT 120 mins (mmol)	8.48	1.00	11.38	2.42	10.03	1.20	9.62	1.20
Leptin (pg/mL)	3048.7	2647.5	5811.1	137.4	8353.1	4355.4	3095.5	2126.3
GLP1 Total (pM)	4.52	1.19	4.16	1.02	4.16	1.15	4.78	2.47
Ghrelin Total (pg/mL)	7141.2	1556.9	3668.2	1219.8	2866.7	2018.9	4745.6	2994.2
FGF21 (pg/mL)	2072.4	778.1	1223	485	2114.3	1152.5	2021.3	1053.9

LD, when compared with HFDw, there is a statistically significant difference, determined via T-test for each metric measured except for total GLP-1. Leptin & OGTT results are elevated for HFDw, whereas Ghrelin & serum FGF21 levels are elevated for LDw. In contrast with this, HFDw vs HFDf group, FGF21 (P=0.028) is the only statistically significant metric, determined with T-test & Mann-Whitney U tests (FGF21 is non-normally distributed for HFDf so Mann U Whitney test is used, for all other groups T-tests are used). FGF21 is expressed in a much higher quantity (2114 pg/mL) on average in a HFDf diet when compared to HFDw (1223 pg/mL). HFDa when compared with HFDw, demonstrates a statistically significant difference for Baseline Insulin, Glucose T-120, & Leptin. Leptin here is drastically lower than the measurements of either HFDw (5881 pg/mL) & HFDf (8353 pg/mL).

3.2 Weight metric comparison

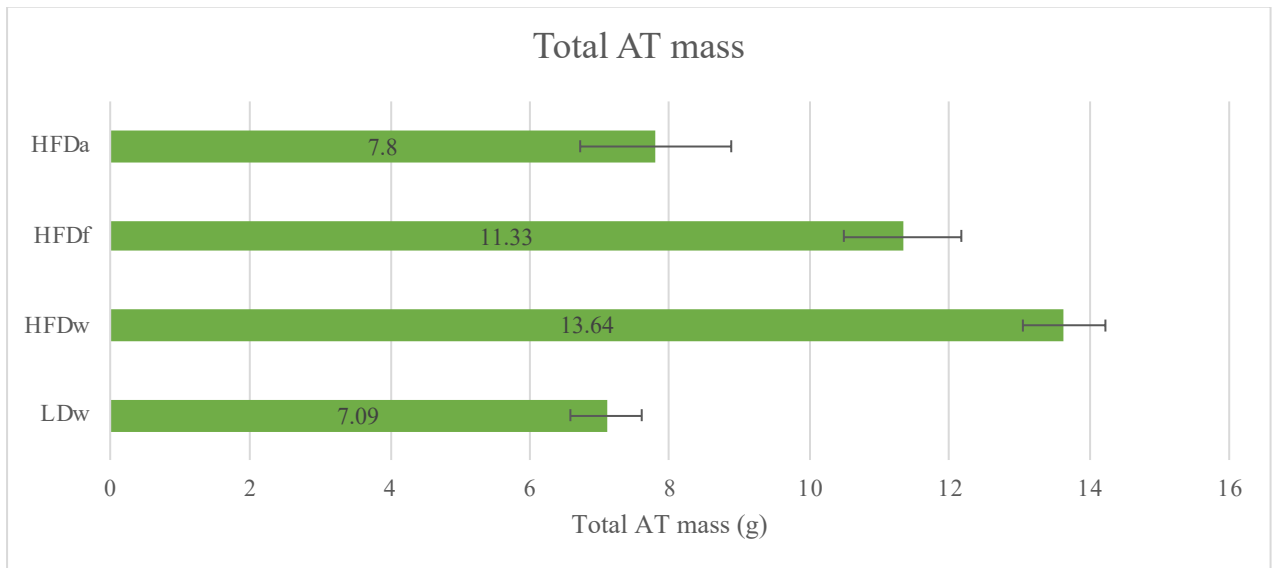


Figure 7: Graphical representation of Total AT mass across groups.

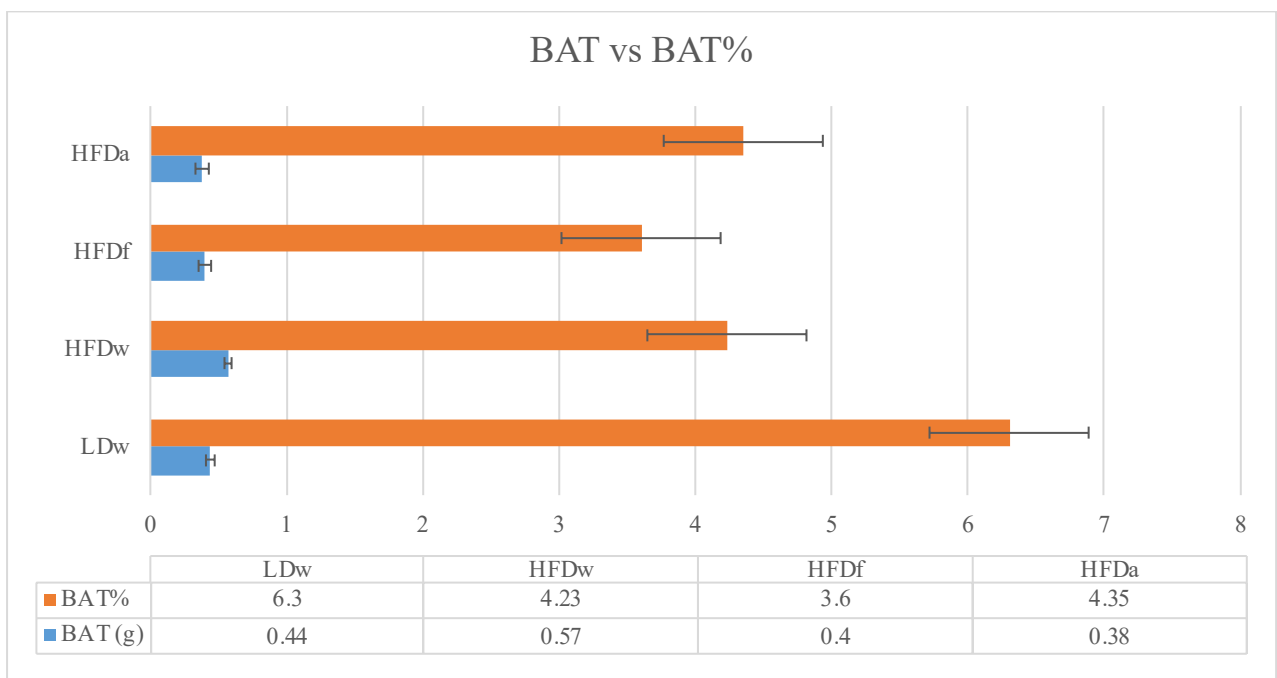


Figure 8: Graphical representation of BAT & %BAT across groups.

Brown Adipose Tissue is a small fraction of overall adipose tissues but is highly important for energy metabolism and thermogenesis. HFDw mice experience a marked increase in BAT amount when compared to LD. Conversely, these mice have a lesser proportion of BAT from

the gross total of AT, however this is due to the large increase in WAT, as shown by Figure 6 above. HFDA mice have a vastly reduced quantity of total AT when compared to both HFDw & HFDf respectively. Both HFDf & HFDA show similar (and statistically insignificant) amounts of BAT. However, both diets are statistically different from HFDw, experiencing a reduction in total BAT. These levels are similar to LDw. Furthermore, BAT proportion of total AT (BAT%) raises slightly in HFDA mice, however this is not statistically relevant. Despite similar levels of BAT, HFDA mice have far less total AT, which is explanatory of the increase in BAT proportion comparing HFDf to HFDA. HFDw vastly increases total AT, but also causes an increase in BAT when compared to both experimental diets.

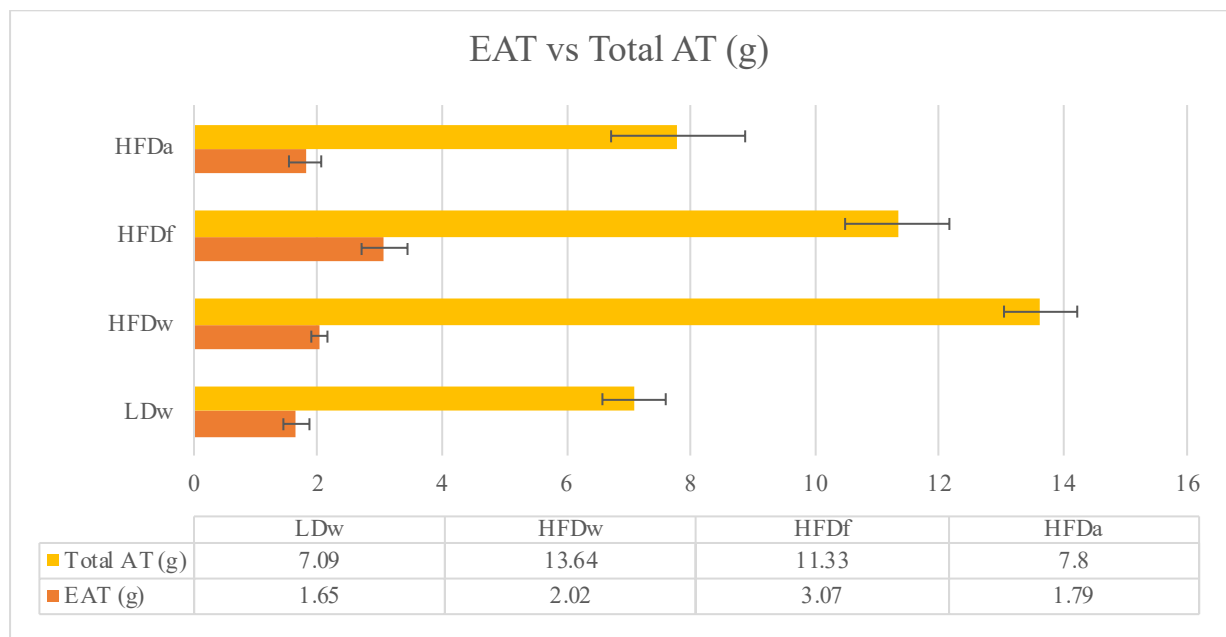


Figure 9: Graphical representation of EAT & Total AT mean weight across groups.

HFDf mice are the only group to have a different EAT quantity, when in comparison to remaining HFD's. HFDf is also statistically different to LD, as there is no statistical difference between HFDw & LD. HFDA also reduces EAT to similar levels to LD & HFDw. Fructose, when added to HFD, appears to exacerbate weight gain across WAT deposits, but has a slight

effect on reducing BAT. HFDA generally reduces AT gain to “normal” levels, although it does not have much effect on BAT levels overall.

3.3 EAT RNA Fold Change.

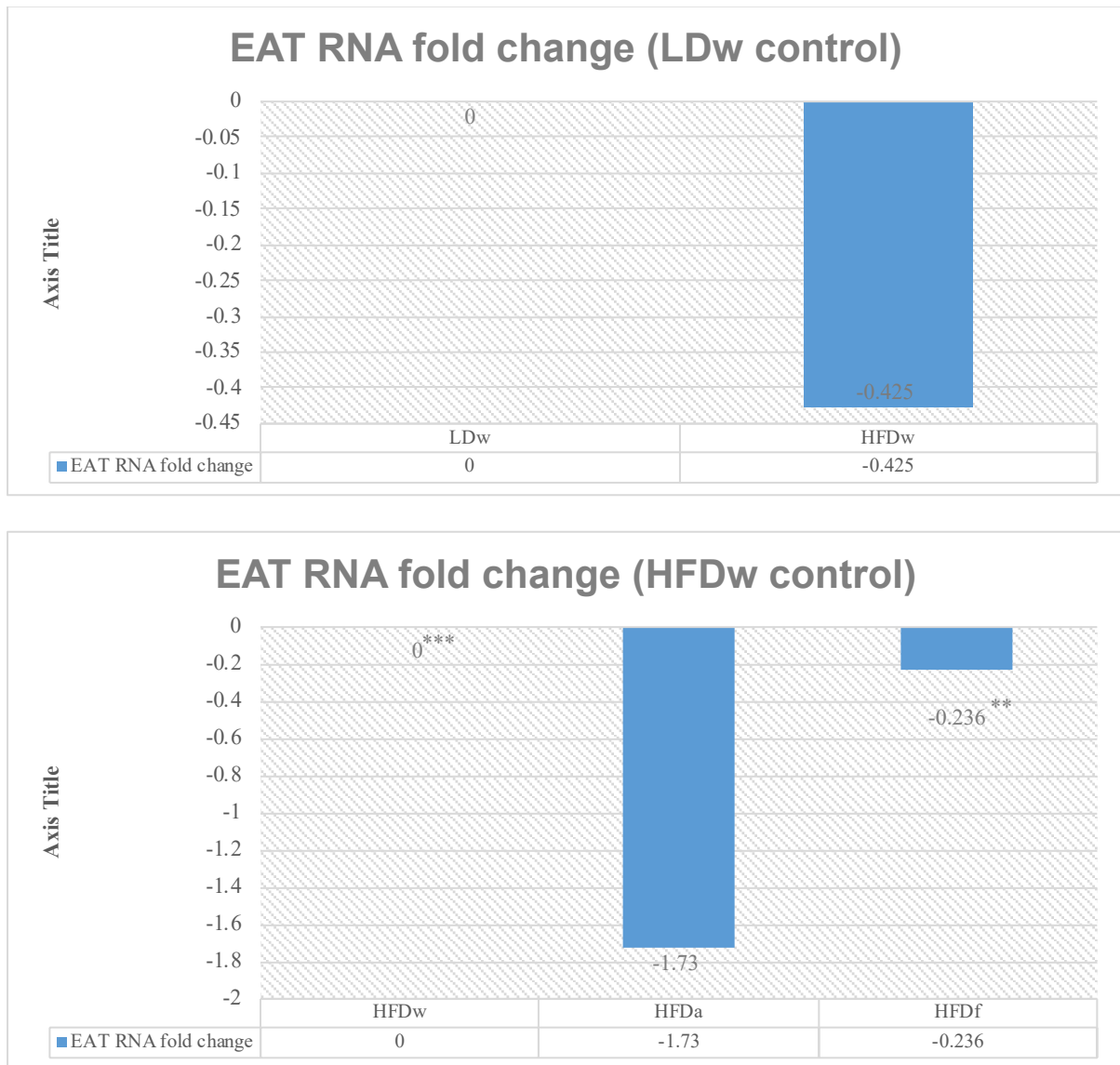
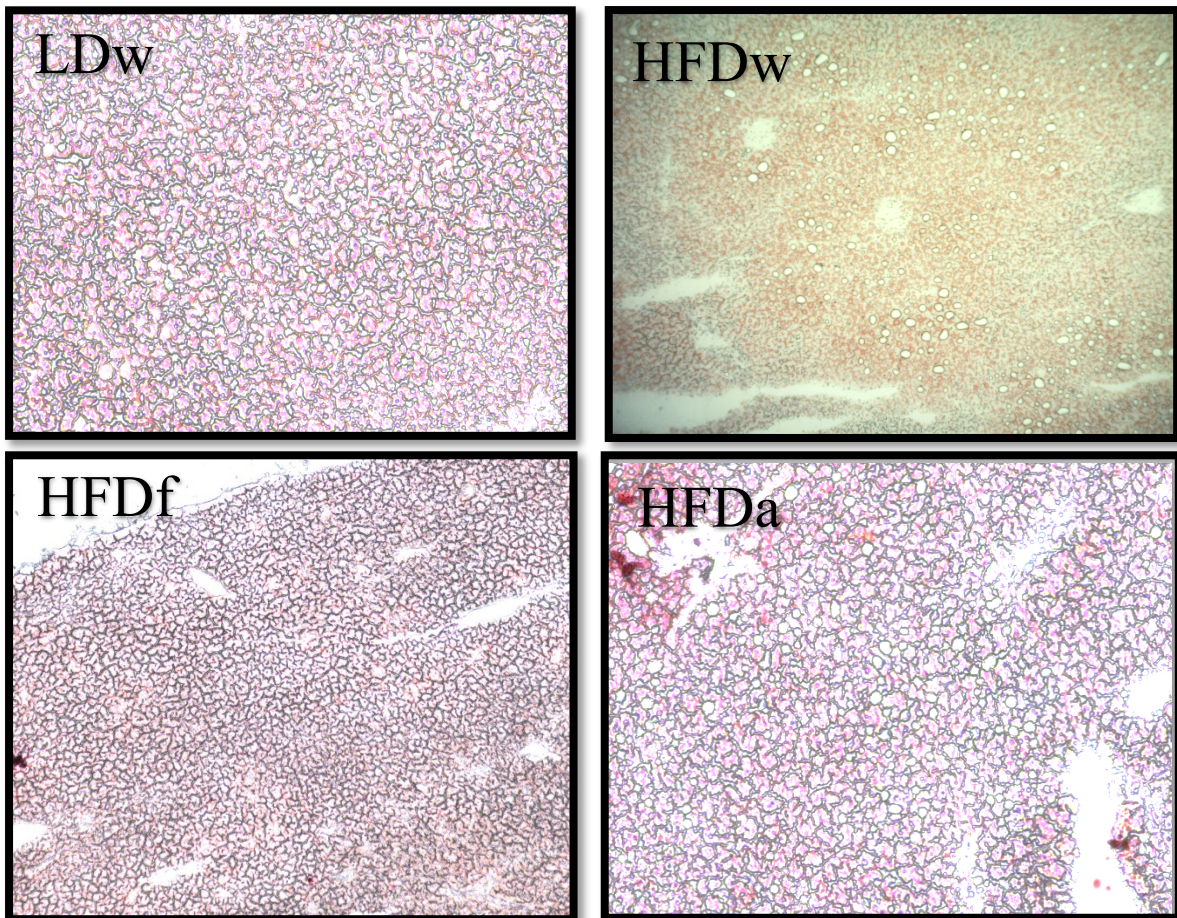


Figure 10: Graphical representation of EAT FGF21 RNA expression across all four diets, generated using the $2^{-\Delta\Delta CT}$ formula, these metrics were then compared utilising the following formula: $Log(Experimental/Control[2])$, which generates the fold-change metric (**listed in Appendix**). There is a 42% reduction in RNA between LDw & HFDw. The addition of Fructose to HFD induces a 23% reduction in FGF21 when compared to HFDw. The addition of Allulose to HFD induces a 170% reduction when compared to HFDw. Mann-Whitney U tests were performed to assess the statistical significance between these metrics, as allulose &

fructose are noted to be non-normally distributed overall ($P=0.038$, $P=0.027$). HFDw is notably indistinct from LDw ($P=0.24$), and similarly, HFDw is indistinct from HFDf ($P=0.7$).

3.4 Histology of Liver samples



LDw, HFDw, HFDf & HFDA samples with ORO stain.(top to bottom, L-R)

Figure 11: Image generated from EVOS M5000 microscope of ORO histological stain applied to mouse liver tissue. Bright red areas are indicative of lipid accumulation, as the non-polar stain binds to the triglyceride molecules present. Hematoxylin stain was also applied, producing the magenta/purple areas, indicative of cell nuclei.

Two liver samples from each diet group were chosen for histological analysis. There was a visible increase in lipid density in HFDf & HFDw mice, contrasted against the similar levels of the HFDA & LDw mice slides. ORO: Oil Red O histological stain.

3.5 Metabolic correlations

Table 2: Summary of HFDw metabolic correlations

HFDw		
Correlation:	Significance (P value):	Pearson Coefficient:
Ghrelin/ OGTT 15 mins	0.038	-0.695
OGTT 15 mins/ FGF21	0.024	0.735

Table 3: Summary of HFDf metabolic correlations

HFDf		
Correlation:	Significance (P value):	Pearson Coefficient:
FGF21/ Brown	0.043	-0.592
Leptin/ Ghrelin	<0.001	-0.824
Leptin/ Brown %	<0.001	-0.849
Leptin/ OGTT 15 mins	0.006	0.735
Leptin/ OGTT 15 mins	0.012	0.695
Ghrelin/ Brown %	0.004	0.761
Ghrelin/ OGTT 15 mins	0.005	-0.746
Ghrelin/ ΔCT	0.047	-0.582
Ghrelin/ ΔΔCT	0.048	-0.58
Brown %/ OGTT 15 mins	0.014	-0.685
OGTT 60 mins/ Total AT	0.032	0.618

Table 4: Summary of HFDA metabolic correlations

HFDA		
Correlation:	Significance (P value):	Pearson Coefficient:
Leptin/ EAT	<0.001	0.926
Leptin/ Total AT	0.003	0.805
Ghrelin/ EAT	0.045	-0.613
Brown/ OGTT 15 mins	0.01	0.733
Brown/ OGTT 30 mins	0.007	0.757
Brown/ OGTT 120 mins	0.009	0.742
Total AT/ OGTT 15 mins	0.007	0.753
Total AT/ OGTT 30 mins	0.002	0.813
Total AT/ OGTT 60 mins	0.02	0.684
Total AT/ OGTT 120 mins	0.007	0.759
OGTT 120 mins/ ΔCT	0.028	0.658
OGTT 120 mins/ ΔΔCT	0.028	0.658

4.0 Discussion & Analysis

FGF21 is a multifaceted “pleiotropic” hormone whose expression is influenced by various factors. Particularly in rodent models, FGF21’s expression and downstream metabolic effects have been well documented (Straub and Wolfrum, 2015).

FGF21 is examined closely for its potential therapeutic effect, demonstrating highly beneficial effects in rodent models, such as reduction in adipose tissue quantity, promotion of insulin sensitivity and glycaemic control. Particularly for hypercaloric diets, weight gain promotes a high level of WAT production, which in turn promotes inflammation, the eventual onset of Diabetes Mellitus II and worsened cardiovascular conditions. FGF21 is shown consistently to promote weight loss, improving overall metabolic conditions (Straub and Wolfrum, 2015). Macronutrient stress and food additives have been continuously identified as particularly influential on FGF21 expression & upregulation across animal models, and in humans to a more limited degree. Protein-restricted, high carbohydrate & high fat content diets are all of particular note, each factor is noted to promote the increase of FGF21 expression & upregulation in rodent models. As the “starvation” hormone, FGF21 promotes more beneficial dietary choices, such as increased protein and reduced carbohydrate intake.

If dietary choices can not only consider caloric intake and appropriate levels of macronutrients amongst other factors, it must also be considered that FG21 upregulation may also be a generally beneficial factor in dietary formulations, particularly for substitution of “traditional”, full-caloric sweeteners.

At 10% of the total calories of Fructose, D-Psicose, or simply Allulose, may prove to be a worthy substitute for diets, both warranting further investigation and also thorough research of its effectiveness not only on FGF21 production, but also on the whole human metabolome.

4.1 qPCR results & FGF21.

Epididymal Adipose Tissue was chosen for this study as it is noted to function as one of the primary targets of FGF21. EAT is particularly important as it functions not only as a WAT triglyceride deposit, it also is highly important for sexual function, such as the development of the seminiferous tubules, spermatogonia & Sertoli cells. EAT, as a potential production site of FGF21, may produce FGF21 in a autocrine manner, but may also be influential of other metabolic pathways yet unforeseen.

From the $2^{-\Delta\Delta CT}$ -formula, HFDw diets on average demonstrate a 42% decrease in EAT FGF21 RNA when compared to LDw diets (on average). This ranges across 9 total samples, inclusive of 3 sample imputation based on missing data metrics for qPCR. This is a vast reduction in EAT FGF21 RNA amount, where conversely the addition of High Fat content only to the diet *reduces* overall FGF21 expression, at least in a localised fashion. Previous studies are indicative that the addition of High Fat content to diets in mice promote the expression in serum, however this is somewhat reflected by another study in mice, performed by Yamazaki et al., 2016. Here, the HFD & LFD (low fat diet) in question displayed a similar level of Liver FGF21 RNA (Yamazaki et al., 2016). Only under VLCD (very low carb. Diet) was liver expression robustly upregulated, approximately ~2.3x fold increase.

The C57/BL6 mice present in this study were subjected to isocaloric diets, suggesting that this under-expression is not unexpected. Solon-Biet et al., 2016 further note that the “primary” macronutrient stresses to promote FGF21 production are protein restriction, in combination with increased carbohydrate levels (Solon-Biet et al., 2016). As a single factor of variation, the addition of High Fat content to the mouse diet is indicated to not induce EAT expression of FGF21. This may be due to FGF21, as previously mentioned, is often called the “starvation” hormone, as particularly in low protein and calorie restricted conditions, it is overexpressed. HFDw diets fed to the C57/BL6 mice are functionally isocaloric (while albeit mouse behavior

may alter total intake), and as such does not induce FGF21 in EAT RNA to the same level of LDw mice.

FGF21 is also noted for its largely liver based expression, however FGF21 levels present in EAT are indicative that it is metabolically induced in EAT also. Similarly, to limited expression in the heart, as described by Cuevas-Ramos et al., 2019, EAT production of this “hepatokine” may also be produced in an autocrine manner, demonstrating localized effects. While both sets of tissues do not function as the primary hormone production site for FGF21, it is readily present in these tissues, as further indicated by the RNA fluctuation present in EAT samples examined here. This may explain the “under-expression” seen in HFDw EAT RNA levels as seen here. Moreover, each qPCR Δ CT metric present in the experimental groups are largely consistent with each other, indicative that total RNA is generally conserved across tissue processing, as such tissue degradation cannot be considered as an influence, instead it is indicative that the fluctuation in RNA levels is entirely diet induced overall.

Furthermore, High Fat Diet + Fructose diet fed mice on average express EAT FGF21 RNA 23% less than even HFDw. This is of note, as “high carbohydrate” proportion of diets are noted to be one of the primary macronutrient stresses associated with FGF21 expression (Solon-Biet et al., 2016). This however is not mirrored by serum expression, where HFDf mice express a slight increase of FGF21 protein in serum (2072 ng vs 2174 ng, $P < 0.05$)

The dramatic reduction in FGF21 RNA in EAT is a noted divergence from literature, however two potential explanations may be considered: negative feedback & hypercaloric intake.

As discussed, general consensus confers that FGF21, particularly in serum expression, is largely induced by increased carbohydrate intake, which is of course indicated by an increase of ~100ng of circulating FGF21 between HFDw & HFDf diet groups. EAT, as a secondary production site of FGF21, may be downregulating overall FGF21 RNA expression in relation

to Liver production, indicated to have an increase by this elevated circulating protein level. This would also be further indicated by a similar, if more severe trend in HFDa EAT levels, where despite a similar serum expression (mean = 2021 ng), EAT RNA levels are vastly downregulated when compared to HFDw, even more so when HFDw is compared to LDw. An alternative, yet not mutually exclusive explanation is that the addition of Fructose to the drinking water of the C57/BL6 mice raised their caloric intake, thus reducing the tissue expression of FGF21. This may also work in conjunction with the aforementioned “negative feedback” to dramatically lower FGF21 expression in EAT. Chapnik et al., 2017 reported an increase in WAT FGF21 protein levels in “restricted feeding” (hypocaloric) diets. Isocaloric, or hypercaloric diets would also indicate reduced levels than standard chow or “lean” diets present in this study. As each RNA level of EAT RNA is standardized against their relative controls, the fold change between LDw & HFDw and HFDw & HFDf respectively, may be a consequence of elevated RNA expression of FGF21 in LDw.

In a similar trend as observed, HFDf EAT RNA sees a 23% loss when directly compared to HFDw levels. This trend is followed by HFDa EAT RNA, which drops a further 170% when in comparison to HFDw. Evidently, diets play a major influence on EAT FGF21 expression. This is somewhat mirrored in a previous study conducted by Hao et al., in 2016. Specifically, Lipid Emulsion (LE) was added to High Carbohydrate diets, where the addition of LE is shown to suppressed overall expression of FGF21 RNA in liver tissue. This could be a result of reduced *de novo* lipogenesis, as fatty acids are more than abundant in the supplied HFD (Hao et al., 2016). LE addition to high sugar content (such as fructose) diets was also shown to downregulate several genes associated with lipogenesis, namely Fatty Acid Synthase (FAS), acetyl-CoA carboxylase (ACC1) and sterol-response element binding protein 1c (SREBPF1) (Hao et al., 2014). This effect is also mirrored in adipose tissue FGF21 RNA, High Carb.

Content alone slightly elevated RNA expression, with a large reduction with the addition of LE (Hao et al., 2016). In support of this, Hao et al., 2016, note that HFD feeding alone reduces overall RNA expression of FGF21(Hao et al., 2016).

HFD's alone when fed to mice reduces liver RNA expression of FGF21, which may correlate with the observed downregulation in EAT observed in section **3.0 Results**, above. The addition of lipid content to High Carb diets are also shown to reduce both liver & WAT expression, due to either the suppression of lipogenic genes, alternatively due to reduced de novo lipogenesis due to the presence of High Fat content in the experimental diet.

This trend also implicates the metabolic action of Allulose: HFDA mice on average experience a ~170% drop in RNA levels when compared to HFDw mice. This is a vast drop in FGF21 expression in EAT, unprecedented by other results seen here, as it is by far the lowest expression across any group. Curiously, this is also mirrored in previous literature: allulose supplementation is linked with increased B-oxidation, in concurrence with a decrease in gross adipose tissue weight, a reduction in the size of adipocytes & a knock-on effect of a reduction in total body weight (Sang Hoon Kim et al., 2017)

Specifically, FGF21 downregulation affects PPAR- γ transcription, which in turn reduces aP2, LPL & FAS, which is further supported by the study by Hao et al., (Sang Hoon Kim et al., 2017). Not only is this correlative with our findings in EAT RNA expression, it is also mirrored in the disparity in total weight gain between each group. HFDA mice developed the second lowest adipose tissue gross weight overall, disregarding LDw. This would imply that the mechanism outlined by Kim et al. is both affecting FGF21, but also total adipose tissue weight as a result of this. The general trend that weight is correlative with FGF21 EAT expression does indeed fit the overall trend seen in our data, with one notable exception: Lean Diet fed mice.

Lean Diet -fed mice have both the lowest adipose tissue mass, but also, conversely, the highest FGF21 expression in tissue.

LDw fed mice have, according to several key markers, the best metabolic state after the 14-week period of the initial study: proportionally high BAT, low weight, & improved OGTT results when compared to HFD's. FGF21 expression may be induced due to malnutrition however, such as hypocaloric intake, as suggested by FGF21's moniker, the "starvation" hormone.

In a study published in 2019 by Fujii et al., FGF21 RNA was observed to be upregulated in WAT deposits of mice fed hypocaloric diets (Calorie Restricted; CR). This is also mirrored in serum expression, promoting robust expression both in serum and tissue (liver & WAT) (Fujii et al., 2019). Aging was also noted to be a factor to influence upregulation, however CR alone upregulated signaling in conjunction with overall expression on *in vitro* adipocytes (Fujii et al., 2019). LDw, despite containing all necessary amino acids, is the least calorie dense of the experimental diets present in our study. When directly comparing FGF21's EAT RNA levels, downregulation in HFD's is not only expected, but directly supported by the appropriate literature; WAT deposits upregulate FGF21 under hypocaloric conditions and downregulate FGF21 under hypercaloric conditions. As LDw is the least nutritive diet here (in conjunction with the subtraction of high lipid content), the control diet for HFDw is relatively high, therefore demonstrating a downwards trend overall.

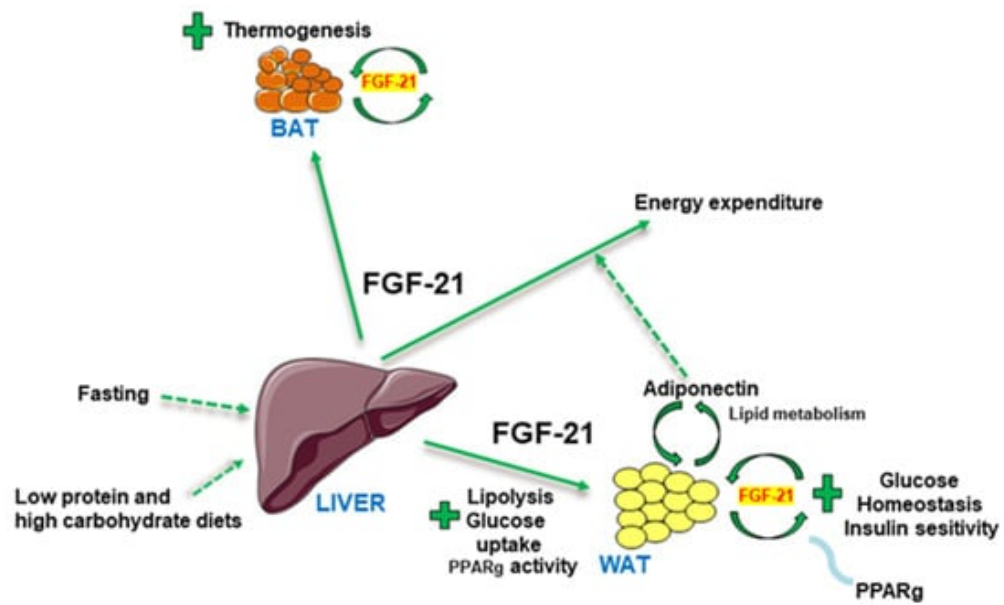


Figure 12: Illustration of upstream influences on FGF21 & downstream effects of FGF21. Broken green arrows on the left represent exogenous influences on FGF21 upregulation: caloric restricted, protein restricted & high carbohydrate content diets. FGF21 is indicated to increase thermogenesis in BAT, increase energy expenditure & act on WAT to increase glucose uptake, insulin sensitivity & PPAR- γ activity (Prida et al., 2022). FGF21: Fibroblast growth Factor 21, BAT: Brown Adipose Tissue, WAT: White Adipose Tissue, PPAR γ : Peroxisome proliferators-activated receptor γ .

4.2 Serum Levels of FGF21

Serum expression of FGF21 is an oft-used metric to analyse the metabolic response to both dietary and other exogenous stimuli, such as administration of therapeutic agents.

There is a vast difference between the LDw mean FGF21 serum expression and that of HFDw (2072ng vs 1223ng, $P < 0.001$). This disparity between groups is indicative of a strong metabolic response to the diets administered to each group. Curiously, the “worst” of these two diets, HFDw, is noted to have vastly reduced expression overall.

One such explanation for this disparity is the change in dietary content: HFDw contains far more lipid content, which has been indicated across literature to help downregulate FGF21 overall (Hao et al., 2016). As discussed previously, this is hypothesized to have two main metabolic triggers: the lack of requirement for *de novo* lipogenesis within the mouse as dietary content provides a more than sufficient amount, and the downregulation of pro-lipogenic genes, specifically FAS, SREBPF1 & ACC1 (Hao et al., 2016). De novo lipogenesis actively promotes liver steatosis, seen in a higher degree in HFDw & HFDf, which would corroborate this hypothetical function of LE addition (Heeren and Ludger Scheja, 2021).

While this effect was primarily observed when lipid-emulsion (LE) was fed in addition to a high carbohydrate diet, it stands to reason that this effect would be mirrored in this experiment, particularly when the baseline level of FGF21 would be decreased as it does not include high carbohydrate content. Both LDw and HFDw diets present are isoenergetic, with no addition to drinking water for mice, as such conditions are virtually identical between groups. This is also a factor to consider with HFDf & HFDa diets. Mice were fed ad libitum, left to their individual discretion for the consumption both of food and drinking water. As the experimental sugar content (Fructose & Allulose) are contained only in drinking water, amounts will differ between each individual mouse and potentially influence the spread of data overall.

Serum levels may also be associated with the hepatic accumulation of lipids, associated with deleterious metabolic disorders and poor diet quality.

In conjunction with this, Fisher et al. posit that obese mice develop “resistance” to FGF21 (Fisher et al., 2010). While both HFDw & HFDF develop very high levels of Adipose tissue respectively, conversely, HFDw serum FGF21 expression is far lower than that of HFDF (1223 ng, $P < 0.001$). Dual-specificity protein phosphatase 5 (DUSP5) is reported to reduce ERK activity and promote hepatocyte death (Ja Hyun Koo et al., 2021). ERK is both a prominent feature of downstream signaling of FGF21, but also reported by Fisher et al., 2010 to have a significant reduction in signaling in Obese mice, suggesting that FGF21 sensitivity is reduced in obese subjects (Fisher et al., 2010). DUSP5 is also reported to be induced by “stress mediated hepatocyte injury” (Ja Hyun Koo et al., 2021), specifically Liver Fibrosis. Diets High in saturated fats and Fructose are also reported to promote Liver fibrosis (Kohli et al., 2010), which would suggest a metabolic link between the addition of fructose to HFD, which would in turn induce DUSP5 by way of liver fibrosis, promoting a decrease in ERK signaling, contributing to FGF21 insensitivity in these mice (Ja Hyun Koo et al., 2021). As outlined by Freeman et al., Diabetic patients suffer with “insulin insensitivity” in which state the body is obliged to overproduce insulin in order to deal with the heightened demand for glucose uptake, as cells become unresponsive to the endogenously produced insulin (Freeman et al., 2023).

We propose that a *potential* mechanism influencing the overall FGF21 level in HFDF mice is the promotion of liver steatosis, leading to the upregulation of DUSP5, the subsequent inhibition of ERK signaling, inducing FGF21 resistance (Ja Hyun Koo et al., 2021).

Liver steatosis between these groups however seems to occur at somewhat similar levels, particularly when analysing Triglyceride density across liver samples, where there is no statistically significant difference. This would infer that if this mechanism did indeed function

in these two groups, then there is a major discrepancy between serum levels, despite somewhat comparable liver steatosis. This may be due to a multitude of reasons, such as “negative feedback” in lieu of full FGF21 insensitivity as suggested by Fisher et al., 2010.

To build upon this, this mechanism would also suggest that Allulose would induce similar conditions in these mice, as the serum FGF21 level is similar to that of HFDf (2021 ng, $P < 0.05$). Allulose, if influencing this metabolic pathway, would induce liver steatosis, leading to FGF21 insensitivity and an upregulation in circulating FGF21 levels. This would also explain the increase in adipose tissue in the HFDA mouse group, despite having overall less AT than HFDw, each mouse on average expresses a far higher level of FGF21 in serum.

However, does not correlate with the overall lipid (triglyceride) density within HFDA mice liver tissue, being closer overall to LDw diet mice. This would suggest that it is a different mechanism, similar to that of LDw mice.

Another such explanation for the heightened serum level of HFDA mice is that it is less calorie dense than that of HFDf. As mentioned previously, higher calorie diets trend towards FGF21 downregulation, whereas protein restricted, high carbohydrate or caloric restricted diets trend towards FGF21 induction. This trend is also exemplary of the reduced weight gain in HFDA mice, particularly when compared to HFDf & HFDw groups. Allulose is one tenth of the total calories of Fructose, which would explain a large disparity between weights. However, this is contrasted by the highest overall weight in HFDw mice, suggesting that caloric intake is not the only factor influencing the metabolic conditions of these mice. This reduction in caloric intake is indicated to be one of the primary reasons for the upregulation of FGF21 in serum, as the proposed DUSP5/ERK signaling mechanism has no apparent metabolic implications on the lipid density of HFDA fed mice.

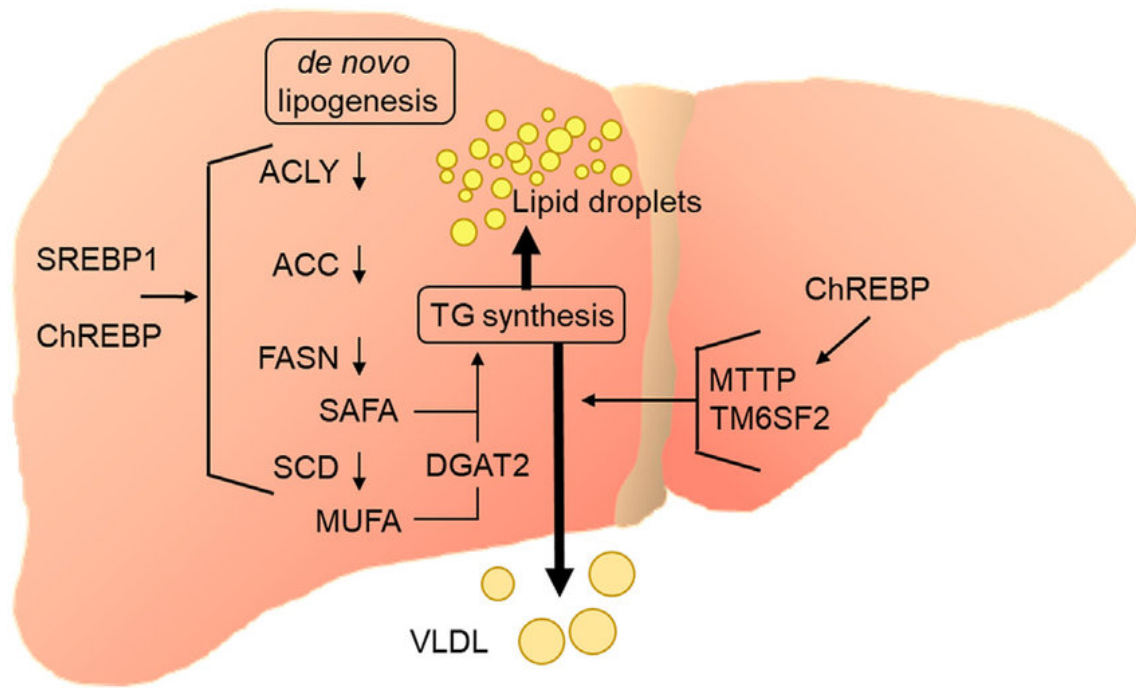


Figure 13: Illustration of de novo lipogenesis within the liver. De novo lipogenesis leads to the development of TG content within the liver, promoting both lipid accumulation & the release of VLDL (Heeren and Ludger Scheja, 2021).

TG: Triglyceride, VLDL: Very Low Density Lipoproteins.

4.3 Histological Analysis

Tissue Processing

Triglyceride content was analysed through the use of ELISA quantification of blood serum & histological analysis. ELISA's were conducted as part of the initial 2019 study, contrasted with the histological analysis of mouse liver in 2023.

As a consequence of the cryogenic preservation and cutting of mouse liver slides, some tissue degradation occurred across a range of slides, amalgamating in two main issues; mechanical tearing of tissue sections and freeze-thaw damage which occurred due to the extreme difficulty in maintaining a continuous temperature of the liver tissue. Tissue samples were cryogenically preserved at extremely low temperatures (-80C) before being fixed in Formalin at -4C overnight, followed embedding within paraffin wax for cutting. Tissue samples, despite chemical preservation, are still sensitive to temperature fluctuation, as such freeze-thaw is nigh unavoidable within any given sample under these conditions. Mechanical tears had occurred during transferral of liver tissue to the glass slides, where slight force on the tissue caused tearing, however overall integrity was preserved.

Due to chemical preservation, tissue samples are, while not exemplary, highly preserved, particularly for lipid content.

ORO staining

ORO staining produced some interesting and varied results between each of the dietary groups present. HFdf was visibly the most affected by lipid accumulation, demonstrating large swathes of red droplets throughout the tissue. This is in-line with previous research, as it is well documented issue, particularly with Western, high fat, high sugar diets.

Across both rodent and human systems, excessive, hypercaloric fructose consumption promotes Non-Alcoholic Fatty Liver Disease (NAFLD) (Kasper et al., 2017). HFdf diet

provided to the mouse subjects is not isocaloric, it is hypercaloric with consumption of fructose, added to the drinking water of the mice. Catabolic breakdown of fructose also produces precursor molecules essential to gluco- & lipogenesis, in addition to catabolism also producing molecules which regulate the transcription of both processes (Kasper et al., 2017).

Furthermore, Calorie restricted diets are indicated to decrease lipid accumulation in liver tissue, whereas hypercaloric diets encourage triglyceride accumulation (Pereira et al., 2012). AIN-93M, the diet fed to mice in a 2012 study by Pereira et al., was constituted by 70% carbohydrate content (Pereira et al., 2012), contrasted with the “standard” Purina chow, at 43.7% carbohydrate content (Pereira et al., 2012). There was a marked increase in lipid accumulation across liver tissue, in conjunction with increased weight and impaired glucose tolerance (OGTT) (Pereira et al., 2012). This is indicative that both (1). Caloric density & (2) Macronutrient stresses are both influential on lipid accumulation.

This is observed in our results, as the HFDf diet fed to the mouse group was both Hypercaloric and contained elevated carbohydrate content. This is further supported by the recorded TG density within liver tissue samples: 6.81 mg/g of liver (mean). This is slightly higher than HFDw and is a vast increase on both HFDA & LDw diet groups. While there is no statistical difference ($P=0.066$) between HFDf and HFDA for the mouse sub-cohort of mice subjected to qPCR, this is statistically distinct for the entire cohort.

HFDw mice, following the general trend observed in this data, express similar levels of lipid accumulation in the liver when compared to HFDf, to a slightly lesser degree, albeit not statistically significant. This is further mirrored in the histological analysis of the liver samples from these mice; there is a comparable level readily visible between samples, seen in **3.0 Results** above, trend towards lipid accumulation is not only readily apparent in this experiment, it is also supported yet again by published literature. One such study by Inoue et al., 2005,

corroborates this, stating that “liver steatosis was induced by overexpression of PPAR γ 1” (Inoue et al., 2005), where High Fat Diet feeding has been noted to induce PPAR γ 1 up-regulation, thus implicit that HFD feeding will lead to the accumulation of TGs within the liver, developing liver steatosis (Inoue et al., 2005). HFDw contains a large amount of dietary lipids, which is noted to contribute to the worsening of liver steatosis, readily apparent both in the visual accumulation of lipid droplets, but also with the density of TG content contained within the liver of the mice as part of this group. At 6.54 mg/g, HFDw mice have the second highest mean of TG liver density, only surpassed by the deleterious metabolic effects of the addition of Fructose.

ORO staining applied to LDw mouse samples produced weaker staining for lipid accumulation. This is also supported by the findings of Pereira et al., where the “standard” Purina pellet diet produced markedly lower levels of lipid expression when compared to carbohydrate overfeeding, hypercaloric diets (Pereira et al., 2012). The addition of High Fat content to diets is also indicated to increase hepatic lipid accumulation (Guilherme et al., 2013). This is further exemplified by the visual difference in lipid accumulation between HFDf & LDw. Fructose addition is also demonstrated to exacerbate this condition, mirroring the HFDf result above (Guilherme et al., 2013). LDw diet is isocaloric, and is considered a “standard” lean chow, as such there is a decreased basal caloric intake. While this is not explicitly “calorie restricted”, it is largely decreased when compared to HFDf, this is further supported by previous literature, as in Pereira et al., 2012, calorie restriction is noted to decrease levels of TG accumulation (Pereira et al., 2012).

Triglyceride density is also correlative with the visual amount of lipid content in the ORO-stained slides, where LDw mice on average have 4.95 mg/g in liver tissue. This is the second lowest average figure, only surpassed by one group: HFDa. Both HFDw & HFDf are elevated

in comparison to both LDw & HFDA, where HFDF mice develop the highest concentration of TG content, which is visibly apparent in our slides above.

There is a trend emerging from both our experimental results & previous studies, where caloric intake & carbohydrate content generally correlates with hepatic lipid content.

This has one notable major divergence: the addition of Allulose to HFD.

HFDA, despite being hypercaloric and also containing high fat content, has visually comparable staining results to LDw. Although this initially appears to be in contrast with previously established norms, (and a major break in the overall trend) Allulose evidently promotes some mechanism of action which largely prevents over accumulation of TGs in the liver tissue. Interestingly, this is a noted finding of a previous study, Itoh et al., 2015. In genetically obese mice (who are predisposed to develop obesity), the addition of Allulose to the standard food pellet, while remaining isocaloric, is noted to promote a decrease in total adipose tissue mass, alongside a notable decrease in liver lipid content (Itoh et al., 2015). This is further indicative of a separate mechanism of action in which Allulose promotes this change. Choi et al., 2018 posit that PGC1 α RNA expression is upregulated in adipocytes when mice are fed Allulose as part of their diet (Choi et al., 2018). This would in turn reduce serum expression of TG's, which in turn would reduce the overall amount of TG's present within the liver (Choi et al., 2018). This is further corroborated by Vega, Huss and Kelly, 2000, who further posit that PGC1- α upregulated Fatty Acid Oxidation (FAO) enzymes, namely LCAD, MCAD, CPT-1 & PPAR- α .

Not only would an increase in FAO increase overall energy expenditure, particularly in tissues such as BAT, leading to overall weight loss, FAO would decrease the overall load of TG's within the blood, in turn leading to a reduction in TG content within the liver itself.

TG liver density is also in support of this mechanism: HFDA has the lowest density of any group present. At 4.64 mg/g, the addition of Allulose is very much apparent in its metabolic effect, and evidently has potent downstream effects on the liver (alongside several other metrics).

In all, there is a clear trend amongst the experimental groups present; the addition of High Fat & Fructose leads to an increase in liver steatosis across the groups, however the addition of Allulose despite being in conjunction with a High Fat diet, vastly ameliorates this negative effect. HFDA diet mice experienced the lowest overall level of liver steatosis, both visually and categorically the lowest TG density across any group: 4.64 mg/g. Allulose may upregulate PGC1 α to induce increased FAO, decreasing the TG burden within serum, reducing overall TG content within the liver. This is reduced through the addition of Allulose alone, as the standard HFD & HFDf diets *both* induce liver steatosis, demonstrative that it is the diet, independent of the addition of fructose, which promotes lipid accumulation. Therefore, Allulose supplementation, despite being introduced to a High Fat Diet, robustly reduces TG's in circulation & within the liver, clearly demonstrative that it is Allulose alone that ameliorates this steatosis.

4.4 Weight & other metrics

Adipose tissues can be divided largely into two main forms: White Adipose Tissue (WAT) and Brown Adipose Tissue (BAT). WAT forms the majority of AT, forming the metabolic caloric storage which is utilised in times of starvation. WAT tissue is the largest influence on total AT mass, as it fluctuates dependent largely on dietary content e.g. calorie content.

High Fat Diet + Fructose (HFDf) diet fed to the cohort of C57/BL6 is the highest calorie density between all of the groups present, and unsurprisingly, has a statistically significant increase of total AT mass when compared to HFDw.

This would be largely expected as HFDf present diet used here is hypercaloric, where the additional calories are introduced through both the addition of Fructose to the drinking water itself and the dietary lipid intake. This of course will in turn induce the development of larger WAT deposits, driving up total AT mass.

However, HFDf has the second highest mean for total AT mass, surpassed by HFDw. FGF21 levels must be considered as a potential influence here also. In serum, HFDf mice possess the highest average concentration of FGF21 protein in circulation. FGF21 is noted to increase energy expenditure through non-shivering thermogenesis & FAO, in conjunction with the inhibition of lipogenesis through the inhibition of sterol regulatory binding proteins (Yano et al., 2022). This mechanism of action would function to aid in the prevention of weight gain overall, where the excess FGF21 serum expression may contribute to overall weight difference between HFDf & HFDw.

While EAT tissue expression of FGF21 is significantly reduced, serum expression remains high, implicit that localized EAT expression is irrelevant to the overall tissue homeostasis of the mice. Serum FGF21 expression appears to be affecting the overall weight of the mice on average.

Building upon this, HFDw mice have the highest mean value for total AT mass. One such mechanism of action to influence this is the lack of total serum expression FGF21. At 1223 ng (mean), FGF21 serum expression for HFDw is drastically decreased when compared to both HFDf & HFDA. This would suggest that FGF21 plays an inhibitory role in the development of adipose tissue. Not only does FGF21 inhibit the action of SREBP's, (Yano et al., 2022) it also increased total energy expenditure through non-shivering thermogenesis (promoting higher expenditure along the mitochondrial lumen) in conjunction with increased β -oxidation of fatty acids (Yano et al., 2022), which are stored in the form of triglycerides in AT deposits. With such a reduced level of FGF21 expression in serum, HFDw mice are potentially at a severe disadvantage, particularly when considering the increased gain of adipose tissue, which will lead to the promotion of insulin resistance, compounded by the lack of FGF21 overall.

Not only are HFDw group mice subject to increased AT gain, but they also express a lesser amount of leptin (the "satiety" hormone) when compared to HFDf (Nogueiras, 2008). 5811 pg/mL (mean) vs 8353 pg/mL (mean) is a significant reduction, particularly when the HFDw diet is also rich in macronutrients. There is no tradeoff for significant metabolic benefits, these mice are less "satiated", more obese, and also possess similar levels of steatosis in the liver.

Compounding this, HFDw mice also demonstrate impaired OGTT results when compared to HFDf mice. While there is not a vast difference, the average for every metric in HFDw sits above their corresponding values in HFDf mice, showing a higher serum burden of Glucose, alongside an impaired glycaemic response to the glucose stressor as part of OGTT. Every relevant metric here is consistently higher, with a noted elevation in initial response for OGTT 15, where HFDw group has an elevation of 11.98 mmol, compared to that of 11 mmol in HFDf. While this may appear to function counterintuitively, this can largely be attributed to FGF21 serum expression.

FGF21 is currently studied for its potential as a potent therapeutic for several metabolic disorders, notably promoting insulin sensitivity both through the promotion of glucose uptake in several tissues, alongside protection from liver & muscle insulin sensitivity (Camporez et al., 2013). Camporez et al. also performed a similar OGTT, where the effect of FGF21 administration was compared to an inert vehicle, demonstrating similar results to our own; FGF21 administration promotes an increased capacity for glucose tolerance (Camporez et al., 2013). This is further exemplified by a large reduction in insulin response to a stressor, where AUC (area under curve of this response) is noted to be significantly reduced with FGF21 administration ($P < 0.01$) (Camporez et al., 2013). Energy expenditure is yet again noted to be increased with FGF21 administration ($P < 0.01$), with a notable increase in VO_2 , indicative of increased capacity for O_2 circulation, a biomarker for aerobic health (Camporez et al., 2013). Evidently, HFDw mice experience a noted lack of benefit from FGF21 expression, apparent from their increase in total AT, lack of “satiety” and increased insulin resistance.

Weight gain for HFDw mice is in vast contrast to both LDw & HFDA mice cohorts. Lean diet fed mice experience the least overall weight gain, which may be attributed to several factors: no change in macronutrient composition of diet, isocaloric diet, and increased FGF21 expression (when compared to HFDw). Adopted as the “negative” control, LDw is proven to function as the healthiest diet for every metric as seen thus far, particularly for adipose tissue gain. Furthermore, FGF21 serum levels are both elevated in serum & EAT tissue expression. There is one significant drawback of the feeding of LDw however: higher levels of Ghrelin (the “hunger” hormone) and decreased levels of Leptin (the “satiety” hormone) (Nogueiras, 2008). Ghrelin is documented to be negatively associated with total adipose tissue mass (Nogueiras, 2008), and as Leptin is purported to function on an axis-basis with Ghrelin, this effect is corroborated by previous research (Nogueiras, 2008).

As mentioned, FGF21 expression is elevated within LDw mice in serum. As with the elevated levels in HFDf, FGF21 promotes adipose tissue loss, through β -oxidation of fatty acids, alongside thermogenesis (Yano et al., 2022).

LDw also induces the “healthiest” of OGTT results, alongside baseline insulin and baseline glucose. LDw on average has the lowest OGTT results, with no impairment to overall response, with a remarkably low insulin and glucose amount in serum.

The addition of Allulose proves to be a potent metabolic regulator, promoting beneficial effects in the mouse cohort present, even despite being used in conjunction with a High Fat Diet. Despite the deleterious effect of HFD alone, the addition of Allulose promotes a severe reduction in weight gain ($P < 0.001$), comparable to even LDw diet. At a weight increase of only 7.8g, Allulose aids in the reduction of a HFD to near LDw levels (7.09g). This can be outlined through two mechanisms: FGF21 expression (in serum) & PGC1- α upregulation (Choi et al., 2018).

As discussed, prior, FGF21 is a pleiotropic hormone with a multitude of effects, the most important of which accommodate sugar and fatty acid metabolism. Allulose induces an elevation in FGF21 serum expression (when compared to HFDw), returning it to similar levels near HFDf & LDw.

Allulose has also been noted to have a marked effect in diabetic model mice, which would be similar to diet exposed to HFDw present here. Hossain et al. 2015 demonstrate that genetically diabetic mice maintain regular blood and insulin levels, when contrasted against the feeding of a regular diet (Hossain et al., 2015). As genetically diabetic models, these mice are predisposed to develop the deleterious conditions associated with Type II Diabetes Mellitus (T2DM), therefore Allulose is a potent metabolic agent which functions to maintain normal blood sugar metrics (Hossain et al., 2015). This is echoed in our own results, in which OGTT, total AT

mass, & insulin metrics approach the “normal” level of LDw. OGTT results, while not as low compared to LDw, are still noticeably improved when compared against HFDw in particular. Glucokinase is promoted when mice are exposed to Allulose in their respective diet, promoting the translocation of glucokinase, which in turn process glucose molecules in serum, reducing the overall burden within the blood (Hossain et al., 2015).

Furthermore, not only does Allulose induce this improvement, it induces this improvement in a High Fat Diet, which as established, is the most likely to induce negative conditions in these mice. There is another interesting development with the addition of Allulose: Leptin is reduced to similar levels as LDw, whereas Ghrelin is slightly increased when compared to HFDw. Leptin, at 3095 pg/mL, is remarkably low when compared to HFDw, however it is comparable to LDw. Satiety is particularly decreased; however, hunger (ghrelin) is notably an intermediate figure, 4747 pg/mL, albeit higher than 3668 pg/mL of HFDw, it is still a vast improvement over that of LDw, at 7141 pg/mL. While this is not as beneficial as possible, the reduction of Ghrelin is a noted improvement, which is an added benefit considering the wealth of other metabolic boons present post addition of Allulose to HFDw.

Brown Adipose Tissue

Evidently, Adipose Tissue total mass varies wildly between the diets present, however there is less fluctuation between Brown Adipose Tissue amounts (BAT) across the diets present here. LDw mice develop 7.09 g of new AT, where 6.3% is representative of BAT. This is interesting, as FGF21 levels in serum is on near-equal footing between LDw, HFDf & HFDa, where FGF21 is regularly noted to promote the browning of AT (Fisher et al., 2012). However, BAT slightly increased in HFDw, and decreased in HFDf and HFDa overall. Proportionally, LDw recruits the highest amount of BAT, but is low when compared to HFDw.

This begets the question: what is the direct cause and implication of this differing BAT level?

BAT is relatively stable overall, however, as total AT mass fluctuates en masse between each of the diets, this ultimately influences BAT proportionality. Increase in BAT seen in HFDw diet appears to function independently of FGF21 serum expression, as HFDw mice develop the most BAT on average.

This may be due to the lack of, conversely, FGF21 expression in serum. HFDw is the only diet present with a *drastically* reduced serum level of FGF21 protein, at ~1223 ng (mean), a reduction of 800 ng when contrasted with the other diets present. As mentioned previously, FGF21 prevents lipogenesis, therefore its reduction in serum expression shown here may influence the overall BAT observed across the mouse models, mirrored by similar results when examining TG content within the liver (Yano et al., 2022).

However, this increase cannot be solely attributed to FGF21, as HFD's have been noted to promote increases of BAT & EAT in a recent study by Alcalá et al., 2017. Here, both tissue metric & blood glucose metrics increased ($P < 0.05$ & $P < 0.01$, respectively), which further correlates with our results seen prior.

Diet is evidently a strong influence not only on total AT mass, but also BAT levels, where the highest increase of AT is somewhat correlative. FGF21, as it is largely influenced by diet, may be the link between cause & effect here, as the addition of Fructose & Allulose both increase overall serum expression in the tested mice cohort, which correlates with a decrease in total BAT mass.

Echoing this sentiment, PPAR- α is shown to induce browning in HFD (Miranda et al., 2020), not only would the HFDw present here promote total AT (mainly WAT) gain, it also promotes its browning through PPAR- α (Miranda et al., 2020). PPAR- α however, is also reported to function in the promotion of FGF21 upregulation within the liver, which would contrast against the observed results. Therefore, the only concrete link to BAT mass is the effect of Diet. While this may be due to FGF21, the observed results do not correlate with this, as such no solid

conclusion may be drawn between these metrics. This however does not exclude the possibility that the overall metabolic effect of BAT is increased/reduced with total mass, as specific genes must be upregulated for a higher energy expenditure, which would link total AT with BAT *activity*, not BAT mass (Fisher et al., 2012).

Diet self-evidently is shown to be a key influence on BAT expression, where the addition of both Allulose & fructose appear to reduce BAT expression to LDw levels, this is echoed in the metabolic disparity observed between HFD/High Fructose, reported by Miranda et al., 2020. Allulose, as a potent metabolic influence, has also been reported to reduce both total AT & BAT masses in previous studies (Chung et al., 2012). This was also observed upon the addition of Allulose to a HFD, mirrored in the experimental results here, evidently Allulose addition reduces overall adiposity, not specific to any group (Chung et al., 2012). While BAT *mass* is ultimately reduced, this is not mutually exclusive with the thermogenic & metabolic properties of BAT, such as the upregulation of CIDEA & UCP-1 (Fisher et al., 2012). This would also be indicative of not only reduced AT mass overall, but also a reduction in liver steatosis, as B-oxidation is increased (Fisher et al., 2012).

Epididymal adipose tissue

Similarly, to both Total AT & BAT, Epididymal Adipose Tissue (EAT) varies between each of the experimental diets. HFDf mice develop more EAT than any group present ($P < 0.05$ HFDf/HFDw, $P < 0.05$ HFDf/HFDa), where EAT is statistically indistinct between any other of the cohorts. This would imply several different causes, such as (1) Fructose metabolism & (2) Hypercaloric feeding.

While implicated from the experimental results here, Fructose metabolism itself is not a direct cause of EAT mass gain. In a study conducted by Lonzezzi et al. in 2017, High Fructose diet was noted to have no effect on overall EAT mass (Lonzezzi et al., 2017). This was conducted

in relation to a potential therapeutic, testing the anti-inflammatory properties of food additives, where High Fructose diet was adopted as the “negative control”. There is neither any difference in average mass nor any statistical significance, implicit that our results are not due to the catabolic breakdown of glucose when it is added to a diet (Lonzetti et al., 2017).

This would imply that sheer caloric intake is the primary factor influencing EAT development overall, independent even of FGF21 serum expression. As HFDf contains both a deleterious solid food high in Fat, it also contains an elevated number of calories supplied by the addition of Fructose to the drinking water of the mice. While total AT mass is decreased when compared to HFDw (due to FGF21 action), EAT is markedly increased, suggesting independence of any ameliorative factor such as FGF21. LDw is observably the lowest EAT mass group, followed by HFDA, HFDw & HFDf. This break in trend for HFDA, despite containing more calories than HFDw, may be attributed to the overall metabolic effect of Allulose supplementation.

This effect is exemplified by a variety of different downstream effects: increase in glycaemic control, decreased liver steatosis & importantly, reduction in weight specifically total AT mass (Hossain et al., 2015). Allulose supplementation is clear in its overall effect, promoting beneficial downstream effects, and this robustly explains the lack of EAT gain in HFDA mice when compared directly to HFDf or HFDw. Despite introduction in a High Fat diet, Allulose is highly ameliorative against the deleterious effects, particularly for AT development.

4.5 Correlations of note across diets.

Pearson correlation statistical tests were carried out across every metric present between each of the diets. Interestingly, several correlations may appear dependent on the macronutrient & supplement supplied to each dietary group. A summary of these findings is listed above ***in 3.5 Metabolic correlations*** & below in ***Appendix***.

Firstly, LDw fed mice demonstrate three positive correlations with Leptin; EAT, Total AT & OGTT 120 mins. Leptin, the “satiety” hormone (Cui et al., 2017), is primarily produced by Adipose Tissue mass (Picó et al., 2021), therefore this correlation is fully corroborated by previous evidence. AT mass, furthermore, is linked with insulin insensitivity/worsened glycaemic control (Gutierrez et al., 2009), indicated again by the increase in OGTT 120 metric, as observed in the LDw correlations.

HFDw mice display an interesting change in correlations: OGTT 15 mins negatively correlates with Ghrelin (measured in total), and positively correlates with FGF21.

Ghrelin, the “hunger” hormone (Cui et al., 2017), is produced in response to less calorie dense diets, acting to increase intake for the mammal models (Cui et al., 2017). As ghrelin is produced in leaner animals, with less AT mass overall, a negative correlation with OGTT 15 mins is supported by previous evidence, as insulin resistance is a trait of AT gain overall (Gutierrez et al., 2009). OGTT 15 mins is also increased in correlation with FGF21 serum expression, indicative that worsened glycaemic control is correlative with FGF21 protein in serum. This may be due to FGF21 insensitivity, as mentioned previously, increased lipid accumulation in liver may lead to FGF21 ERK/AMPK signaling degradation, leading in turn to decrease in insulin sensitivity (Ja Hyun Koo et al., 2021). This correlation supports the aforementioned results seen in each of the ELISA metrics & visual accumulation of lipid throughout the liver slides.

The addition of Fructose to HFD contributes to a host of new correlations observed across the HFDF group.

Interestingly, FGF21 serum expression is negatively correlated with Brown adipose tissue mass. This initially appears to contrast with previously established literature: FGF21 induces the browning of AT deposits (Fisher et al., 2012), a negative correlation here appears to indicate an opposite of this effect overall. However, insensitivity to FGF21 would explain this quandary, where it cannot effectively brown the white AT mass, and Brown AT mass is overall increased by the hypercaloric diet, as mentioned above in “Weight and other metrics”, therefore increase in BAT, compounded with insensitivity of FGF21 “browning” contributes to this negative correlation. Curiously, HFDF mice somewhat mirror an observed correlation: Leptin correlates with OGTT metrics, specifically 15- & 30-min metrics. This is because Leptin is produced by AT mass (Picó et al., 2021), which is the highest in HFDF mice, as observed in previous results. Leptin and ghrelin are strongly negatively correlated with each other, where an elevated Leptin expression corresponds with a decrease in Ghrelin overall. Leptin, as produced by AT deposits (Picó et al., 2021), is naturally overexpressed in largely obese mice exposed to this diet. Ghrelin, contrastingly to Leptin, is negatively associated with OGTT results – indicative that ghrelin is under expressed when AT mass is increased, as leptin is produced from AT mass (Gutierrez et al., 2009), further supporting the negative correlation between that of Ghrelin & Leptin.

Furthermore, Ghrelin is negatively associated with $\Delta\Delta\text{CT}$, the metric for EAT FGF21 RNA expression, suggesting that adipocyte FGF21 expression is contrasted with overall ghrelin expression. Ghrelin is noted to not be attenuated by the addition of fructose, inhibiting its suppression (Petya Hadzhibozheva et al., 2023). The addition of fructose, despite not contributing the maximal amount of AT mass development, may overall prove deleterious if it

does not inhibit the suppression of Ghrelin, which would lead to overfeeding as Ghrelin is a highly important factor for appetite.

The addition of Allulose to HFD also introduces some further interesting correlations both positive and negative. As expected, Leptin is strongly correlated with both EAT and Total AT metrics, as EAT is a subcategory of white adipocytes, where total AT is largely constituted of WAT, Leptin is produced by AT masses throughout mammalian systems (Gutierrez et al., 2009). Ghrelin here is, unsurprisingly, negatively correlated with EAT, as ghrelin is downregulated by hypercaloric diets, as it acts as the “hunger” hormone, regulating the diet (Cui et al., 2017). This may also be a positive benefit, as ghrelin resistance is a noted pathology of obesity, which leads to difficulty (Farhana Naznin et al., 2015) in maintaining proper levels of caloric intake. This negative correlation, while not particularly beneficial, particularly for the upregulation of Ghrelin with leaner mice, it does indicate normal metabolic function i.e. Ghrelin sensitivity (Farhana Naznin et al., 2015).

Both brown & WAT metrics (total AT etc.) are positively correlated with OGTT metrics, suggesting that glycaemic control is dampened by increases in AT mass, brown or white. This is again supported by previous literature (Gutierrez, Puglisi and Hasty, 2009), this correlation would suggest that typical metabolic function is observed across this diet.

The addition of Allulose appears to induce normal metabolic function, as seen with the correlations which are robustly supported by previously established evidence.

4.6 Experimental Question & summary of findings

There is a multitude of angles of analysis regarding FGF21, its expression, and the implications for its expression level, whether it be through diets, therapeutic intervention, the full metabolic downstream effect, or the sum total of these effects in animal models, and by extension, *H. sapiens*. The rare sugar Allulose is of particular interest, as it is researched to have potent downstream metabolic effects, implicated to influence FGF21 alongside several other hormones expressed by mammalian systems.

Tissue expression of EAT, similar to serum level, is largely influenced by overall environment, exercise and in particular, dietary content. This is best exemplified through the liver expression both seen and discussed in this thesis, where EAT RNA expression of FGF21 was differentially expressed dependent on overall diet.

Lean Diet (LDw) robustly induced the highest level, followed by a ~42% reduction in EAT RNA with HFDw feeding, further reduced by ~23% in HFDf, which is drastically inhibited in HFDa, reduced by 170% (compared to HFDw). This may be due to HFD feeding, as in previous literature it is noted to reduce tissue RNA FGF21 expression (Hao et al., 2016). Dietary lipid intake is shown to downregulate overall tissue expression, not exclusive to adipocytes, and is also correlative with our observed results (Hao et al., 2016).

Notably, LDw is particularly over-expressed (in contrast to this overall trend) however as “lean” diet it is isocaloric without any macronutrient change, so as a baseline reading this FGF21 level may be elevated, particularly when considering Lipid emulsion feeding ameliorates FGF21 expression (Hao et al., 2016). Apparent from these results, EAT is highly metabolically active, where FGF21 expression is differentially expressed dependent on overall dietary intake.

This is in contrast with serum expression of FGF21 where each diet largely induced FGF21 expression, with a notable exception of HFDw, which under-expressed ~800ng (P<0.05). This may be addressed when considering the addition of Lipid emulsion is shown to downregulate FGF21 expression (Hao et al., 2016). This is due to the reduction in de novo lipogenesis, or the inhibition of pro-lipogenic genes, such as SREBP1, FAS or ACC1 (Hao et al., 2014), as it is suggested that lipid content provided by HFDw is more than sufficient for the mouse cohort. Allulose and Fructose diets here are both upregulated (for FGF21 expression), suggesting that the mechanism of sugar catabolism, if not indirect metabolic effects influence the overall FGF21 level. Furthermore, it may develop from “intolerance” as described by Fisher et al., 2010 for the specific case of HFDF-fed mice, where the hepatic stress induced by Fructose feeding would contribute to DUSP5 upregulation, leading to ERK inactivation, therefore inducing FGF21 intolerance (Ja Hyun Koo et al., 2021). This exact mechanism is unclear however it is overall clear that Allulose largely induced serum FGF21 expression across the tested mouse cohort.

This is correlated with observed histological analysis of liver samples, alongside the analysis of Triglyceride (TG) density within the liver of these mice. HFDw & HFDF diets see overly dense TG accumulation within their liver, whereas Allulose addition drops TG density to near-LDw levels. This is suggestive that FGF21 activation of metabolic pathways is only truly effective within the LDw & HFDA diets, as despite an overexpressed level of FGF21, HFDF fed mice experience more severe levels of liver steatosis, further corroborating the “insensitivity” mechanism. As described in Figure 12 above, de novo lipogenesis (which is linked to lower levels of FGF21, alternatively, its lack of efficacy) promotes the development of liver steatosis, which is consistent across both HFDw & HFDF (Heeren and Ludger Scheja,

2021). The addition of allulose not only attenuates this condition, but also reduces the overall effect of HFD, despite its evident negative metabolic effect.

Furthermore, Allulose is also shown to robustly reduce overall AT mass, consistently reducing each AT metric to near “normal” (LDw) levels. This is contrasted against the levels of both HFDw & HFDf. Specifically, HFDw is shown to robustly induce the development of both BAT & WAT deposits, largely increasing overall weight, which of course induces risk for the development of Diabetes Mellitus Type II (DMT2), cancer, amongst other related disorders. HFDf sees a slight attenuation, with a notable increase in EAT overall when compared to HFDw. Allulose addition largely ameliorates the effect of HFD, working through mechanisms such as FGF21 to reduce AT levels. FGF21 specifically is associated with increased energy expenditure within BAT, increased FAO & thermogenic activity (Yano et al., 2022), increasing baseline metabolic rate, decreasing overall weight in mice such as HFDa & LDw, which both experience elevated FGF21 levels.

The addition of allulose to HFD also appears to return metabolic functions to “normal” states, as there is a significant number of correlations which suggest typical healthy metabolic function: i.e. OGTT increase associated with higher AT mass, Leptin is associated with AT mass. This contrasts with the correlations seen in HFDf, such as Ghrelin associated with OGTT: implicating worsened glycaemic control in conjunction with ghrelin “insensitivity” (also observed in previous evidence) (Farhana Naznin et al., 2015). While it is not efficient to solely analyse the effect of Allulose supplementation merely through this set of statistical tests, it does further suggest the overall positive effects of Allulose supplementation when added to the HFD’s.

There is one drawback with the addition of HFDA however, and this is the reduction in Leptin, the “satiety” hormone, and the increase in Ghrelin “the hunger hormone” when compared to both HFDw & HFDf. While this is a noted improvement when compared to LDw, which respectively induces higher Ghrelin & lower Leptin levels, this is still a negative effect, as it can promote overfeeding. However, obesity is observed to promote resistance to Leptin, so it’s particularly high expression here may be attributed to the highly obese mice in HFDw & HFDf cohorts (Cui et al., 2017). Interestingly, FGF21 signals to glutamatergic neurons, which is vital for the promotion of weight loss (Claflin et al., 2022). The over-expression of Leptin in HFDw & HFDf and their subsequent insensitivity seems to be correlated with increased weight levels, as described by Claflin et al., 2022. This suggests that FGF21 has an important role in weight loss and its sensitivity in mammalian systems is highly important for both homeostasis and promotion of Leptin sensitivity (Claflin et al., 2022).

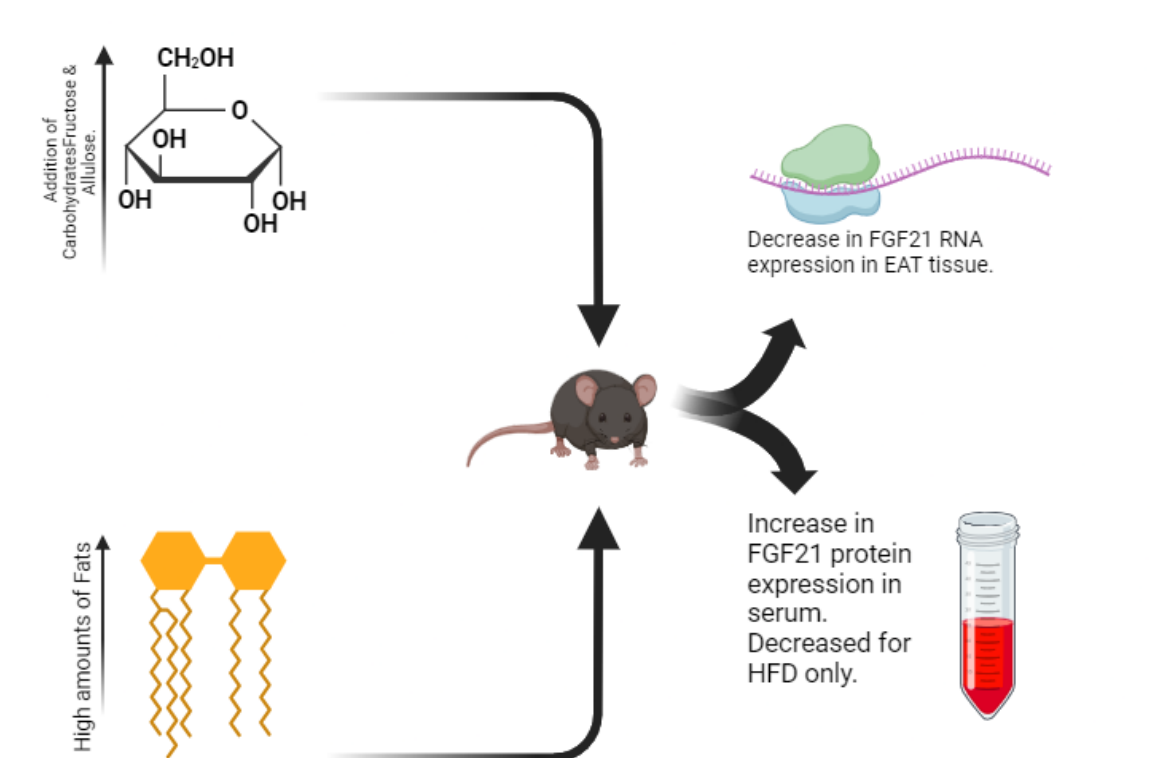


Figure 14: Illustration of summary effect of HFD feeding on FGF21 tissue & serum levels (Created with Biorender.com).

5.0 Conclusion

Overall, Allulose has a distinct and readily apparent effect on several metabolic metrics observed in HFD fed mice.

Allulose supplementation reduces weight gain, reduces OGTT results (therefore improving insulin sensitivity), decreases liver steatosis, and promotes Leptin sensitivity.

FGF21 serum expression is upregulated when HFD is supplemented with Allulose and is robustly downregulated with this supplementation. This is both supported by literature, and correlative with a variety of our experimental results as explained prior.

FGF21 expression within tissue, specifically EAT, appears to have no readily apparent effect on the C57/BL6 mouse model, as the vastly reduced expression of FGF21 in EAT RNA does not inhibit the overall action of FGF21, particularly when considering AT mass, insulin sensitivity, or energy expenditure.

When contrasted against both HFDf & HFDw (with its respective control LDw), the addition of Allulose to HFD allows for the amelioration of several negative health effects, whilst having the added benefit of a reduced caloric intake, as it is a fraction of “traditional” sweeteners such as Glucose or Fructose. This overall benefit is conferred regardless of EAT RNA expression, indicative that it is serum expression that is the primary influence on the metabolic state of these mice. While limited in its overall scope, these experiments have allowed for further research into the full metabolic implications of Allulose, alongside the analysis on its effect on FGF21 expression in white adipocyte tissue. As outlined above, the hypothesis of this thesis is: *“Allulose supplementation alters the expression of FGF21, both in adipocyte & serum expression, leading to the promotion of several beneficial metabolic effects”*, this is robustly demonstrated by the wealth of experimental data, and as such, Allulose shows promise as a food additive, particularly for its addition to “unhealthy” high fat diets, where it is shown to ameliorate the worst effects of this macronutrient stress.

Looking forward, research must be undertaken to fully understand its metabolic effect, but there is vast potential for its use, particularly when considering its effect on the pleiotropic peptide hormone, Fibroblast Growth Factor 21.

References:

- Ahmed, A., Khan, T., D. Dan Ramdath, Cyril W.C. Kendall and Sievenpiper, J.L. (2021). Rare sugars and their health effects in humans: a systematic review and narrative synthesis of the evidence from human trials. *Nutrition Reviews*, [online] 80(2), pp.255–270. doi:<https://doi.org/10.1093/nutrit/nuab012>.
- Annual Reviews. (2016). *Understanding the Physiology of FGF21*. [online] Available at: <https://www.annualreviews.org/doi/10.1146/annurev-physiol-021115-105339> [Accessed 19 Dec. 2023].
- Berglund, E.D., Li, C.Y., Bina, H.A., Lynes, S.E., M. Dodson Michael, Shanafelt, A.B., Alexei Kharitonov and Wasserman, D.H. (2009). Fibroblast Growth Factor 21 Controls Glycemia via Regulation of Hepatic Glucose Flux and Insulin Sensitivity. *Endocrinology*, [online] 150(9), pp.4084–4093. doi:<https://doi.org/10.1210/en.2009-0221>.
- BonDurant, L.D., Ameka, M., Naber, M.C., Markan, K.R., Idiga, S.O., Acevedo, M.R., Walsh, S.A., Ornitz, D.M. and Potthoff, M.J. (2017). FGF21 Regulates Metabolism Through Adipose-Dependent and -Independent Mechanisms. *Cell Metabolism*, 25(4), pp.935-944.e4. doi:<https://doi.org/10.1016/j.cmet.2017.03.005>.
- Bourdon, G., Estienne, A., Chevaleyre, C., Christelle Ramé, Fabrice Guérif, Brun, J.-S., Vasseur, C., Gaëlle Fromont, Plotton, I., Dufour-Rainfray, D., Caldas-Silveira, E., Dupont, J., Froment, P. and Pierre-Henri Ducluzeau (2022). The Hepatokine FGF21 Increases the Human Spermatozoa Motility. *Frontiers in Endocrinology*, [online] 13. doi:<https://doi.org/10.3389/fendo.2022.775650>.
- Carolline Santos Miranda, Flávia Maria Silva-Veiga, Fabiane Ferreira Martins, Tamiris Lima Rachid, Carlos Alberto Mandarin-de-Lacerda and Souza-Mello, V. (2020). PPAR- α activation counters brown adipose tissue whitening: a comparative study between high-fat– and high-fructose–fed mice. *Nutrition*, [online] 78, pp.110791–110791. doi:<https://doi.org/10.1016/j.nut.2020.110791>.
- Caunt, C.J. and Keyse, S.M. (2012). *Dual-specificity MAP kinase phosphatases (MKPs): Shaping the outcome of MAP kinase signalling*. [online] ResearchGate.

Available at: https://www.researchgate.net/publication/229425205_Dual-specificity_MAP_kinase_phosphatases_MKPs_Shaping_the_outcome_of_MAP_kinase_signalling [Accessed 12 Dec. 2023].

- Chen, Z., Yang, L., Liu, Y., Huang, P., Song, H. and Zheng, P. (2022). The potential function and clinical application of FGF21 in metabolic diseases. *Gastrointestinal and Hepatic Pharmacology*, [online] 13. doi:<https://doi.org/10.3389/fphar.2022.1089214>.
- Choi, B.-R., Kwon, E., Kim, H.-J. and Choi, M. (2018). Role of Synbiotics Containing d-Allulose in the Alteration of Body Fat and Hepatic Lipids in Diet-Induced Obese Mice. *Nutrients*, [online] 10(11), pp.1797–1797. doi:<https://doi.org/10.3390/nu10111797>.
- Chu, Y., Huddleston, G.G., Clancy, A.N., Ruth and Bartness, T.J. (2010). Epididymal Fat Is Necessary for Spermatogenesis, but not Testosterone Production or Copulatory Behavior. *Endocrinology*, [online] 151(12), pp.5669–5679. doi:<https://doi.org/10.1210/en.2010-0772>.
- Claflin, K.E., Sullivan, A.I., Naber, M.C., Flippo, K.H., Morgan, D.A., Neff, T.J., Jensen-Cody, S.O., Zhu, Z., Zingman, L.V., Kamal Rahmouni and Potthoff, M.J. (2022). Pharmacological FGF21 signals to glutamatergic neurons to enhance leptin action and lower body weight during obesity. *Molecular Metabolism*, [online] 64, pp.101564–101564. doi:<https://doi.org/10.1016/j.molmet.2022.101564>.
- Cuevas-Ramos, D., Mehta, R. and Aguilar-Salinas, C.A. (2019). Fibroblast Growth Factor 21 and Browning of White Adipose Tissue. *Frontiers in Physiology*, [online] 10. doi:<https://doi.org/10.3389/fphys.2019.00037>.
- Cui, A., Hu, Z., Han, Y., Yi Ching Yang and Liu, Y. (2017). Optimized Analysis of In Vivo and In Vitro Hepatic Steatosis. *Journal of Visualized Experiments*, [online] (121). doi:<https://doi.org/10.3791/55178>.
- Cui, H., López, M. and Kamal Rahmouni (2017). The cellular and molecular bases of leptin and ghrelin resistance in obesity. *Nature Reviews Endocrinology*, [online] 13(6), pp.338–351. doi:<https://doi.org/10.1038/nrendo.2016.222>.

- Dai, B., Xu, R., Li, X., Huang, L., Hopkins, C., Wang, H., Yao, H., Mi, J., Zheng, L., Wang, J., Tong, W., Ho, D., Li, Y., He, X., Hu, P., Chen, Z., Zu, H., Li, Y., Yao, Y. and Jiang, Q. (2022). Macrophages in epididymal adipose tissue secrete osteopontin to regulate bone homeostasis. *Nature Communications*, [online] 13(1). doi:<https://doi.org/10.1038/s41467-021-27683-w>.
- Ding, X., Boney-Montoya, J., Owen, B.M., Bookout, A.L., Coate, K.C., Mangelsdorf, D.J. and Kliewer, S.A. (2012). β Klotho Is Required for Fibroblast Growth Factor 21 Effects on Growth and Metabolism. *Cell Metabolism*, [online] 16(3), pp.387–393. doi:<https://doi.org/10.1016/j.cmet.2012.08.002>.
- Echeverría, F., Valenzuela, R., Bustamante, A., Álvarez, D., Macarena Ortíz, Espinosa, A., Illesca, P., Gonzalez-Mañán, D. and Videla, L.A. (2019). High-fat diet induces mouse liver steatosis with a concomitant decline in energy metabolism: attenuation by eicosapentaenoic acid (EPA) or hydroxytyrosol (HT) supplementation and the additive effects upon EPA and HT co-administration. *Food & Function*, [online] 10(9), pp.6170–6183. doi:<https://doi.org/10.1039/c9fo01373c>.
- Escoté, X., Félix-Soriano, E., Gayoso, L., Huerta, A., María Antonella Alvarado, Ansorena, D., Iciar Astiasarán, J. Alfredo Martínez and Moreno-Aliaga, M.J. (2018). Effects of EPA and lipoic acid supplementation on circulating FGF21 and the fatty acid profile in overweight/obese women following a hypocaloric diet. *Food & Function*, [online] 9(5), pp.3028–3036. doi:<https://doi.org/10.1039/c8fo00355f>.
- Fan, X., Yao, H., Liu, X., Shi, Q., Liang Lv, Li, P., Wang, R., Tang, T. and Qi, K. (2020). High-Fat Diet Alters the Expression of Reference Genes in Male Mice. *Frontiers in Nutrition*, [online] 7. doi:<https://doi.org/10.3389/fnut.2020.589771>.
- Farhana Naznin, Takehiko Koji, T.M. Zaved Waise, Namkoong, C., Abu, Sakoda, H. and Nakazato, M. (2015). Diet-induced obesity causes peripheral and central ghrelin resistance by promoting inflammation. *Journal of Endocrinology*, [online] 226(1), pp.81–92. doi:<https://doi.org/10.1530/joe-15-0139>.
- Fisher, F.M., Chui, P.C., Antonellis, P.J., Bina, H.A., Alexei Kharitonov, Flier, J.S. and Eleftheria Maratos-Flier (2010). Obesity Is a Fibroblast Growth Factor 21

(FGF21)-Resistant State. *Diabetes*, [online] 59(11), pp.2781–2789.

doi:<https://doi.org/10.2337/db10-0193>.

- Fisher, ffolliott M., Kim, M., Doridot, L., Cunniff, J.C., Parker, T.S., Levine, D.M., Hellerstein, M.K., Hudgins, L.C., Maratos-Flier, E. and Herman, M.A. (2017). A critical role for ChREBP-mediated FGF21 secretion in hepatic fructose metabolism. *Molecular Metabolism*, 6(1), pp.14–21.
doi:<https://doi.org/10.1016/j.molmet.2016.11.008>.
- Fisher, ffolliott M., Kleiner, S., Douris, N., Fox, E.C., Mepani, R.J., Verdeguer, F., Wu, J., Kharitonkov, A., Flier, J.S., Maratos-Flier, E. and Spiegelman, B.M. (2012). FGF21 regulates PGC-1 α and browning of white adipose tissues in adaptive thermogenesis. *Genes & Development*, [online] 26(3), pp.271–281.
doi:<https://doi.org/10.1101/gad.177857.111>.
- Flippo, K.H. and Potthoff, M.J. (2021). Metabolic Messengers: FGF21. *Nature Metabolism*, 3(3), pp.309–317. doi:<https://doi.org/10.1038/s42255-021-00354-2>.
- Freeman, A.M., Acevedo, L.A. and Pennings, N. (2023). *Insulin Resistance*. [online] Nih.gov. Available at: <https://www.ncbi.nlm.nih.gov/books/NBK507839/> [Accessed 7 Dec. 2023].
- Fujii, N., Seira Uta, Kobayashi, M., Sato, T., Okita, N. and Yoshikazu Higami (2019). Impact of aging and caloric restriction on fibroblast growth factor 21 signaling in rat white adipose tissue. *Experimental Gerontology*, [online] 118, pp.55–64.
doi:<https://doi.org/10.1016/j.exger.2019.01.001>.
- Ge, X., Wang, Y., Karen S.L. Lam and Xu, A. (2012). Metabolic actions of FGF21: molecular mechanisms and therapeutic implications. *Acta Pharmaceutica Sinica B*, [online] 2(4), pp.350–357. doi:<https://doi.org/10.1016/j.apsb.2012.06.011>.
- Guilherme, U., Santos, Marcelo Eustáquio Silva, Geraldo, Maria José Campagnole-Santos and Andréia Carvalho Alzamora (2013). Age-dependent effect of high-fructose and high-fat diets on lipid metabolism and lipid accumulation in liver and kidney of rats. *Lipids in Health and Disease*, [online] 12(1).
doi:<https://doi.org/10.1186/1476-511x-12-136>.

- Gutierrez, D.A., Puglisi, M.J. and Hasty, A.H. (2009). Impact of increased adipose tissue mass on inflammation, insulin resistance, and dyslipidemia. *Current Diabetes Reports*, [online] 9(1), pp.26–32. doi:<https://doi.org/10.1007/s11892-009-0006-9>.
- Hao, L., Ito, K., Kuan Hsun Huang, Sudathip Sae-tan, Lambert, J.D. and A. Catharine Ross (2014). Shifts in dietary carbohydrate-lipid exposure regulate expression of the non-alcoholic fatty liver disease-associated gene PNPLA3/adiponutrin in mouse liver and HepG2 human liver cells. *Metabolism*, [online] 63(10), pp.1352–1362. doi:<https://doi.org/10.1016/j.metabol.2014.06.016>.
- Hao, L., Kuan Hsun Huang, Ito, K., Sudathip Sae-tan, Lambert, J.D. and A. Catharine Ross (2016). Fibroblast Growth Factor 21 (Fgf21) Gene Expression Is Elevated in the Liver of Mice Fed a High-Carbohydrate Liquid Diet and Attenuated by a Lipid Emulsion but Is Not Upregulated in the Liver of Mice Fed a High-Fat Obesogenic Diet. *Journal of Nutrition*, [online] 146(2), pp.184–190. doi:<https://doi.org/10.3945/jn.115.216572>.
- He, L., Deng, L., Zhang, Q., Guo, J., Zhou, J., Wen-jian, S. and Yuan, F. (2017). Diagnostic Value of CK-18, FGF-21, and Related Biomarker Panel in Nonalcoholic Fatty Liver Disease: A Systematic Review and Meta-Analysis. *BioMed Research International*, [online] 2017, pp.1–12. doi:<https://doi.org/10.1155/2017/9729107>.
- Heeren, J. and Ludger Scheja (2021). *Metabolic-associated fatty liver disease and lipoprotein metabolism*. [online] ResearchGate. Available at: https://www.researchgate.net/publication/351017725_Metabolic-associated_fatty_liver_disease_and_lipoprotein_metabolism [Accessed 14 Dec. 2023].
- Herman, M.A. and Birnbaum, M.J. (2021). Molecular aspects of fructose metabolism and metabolic disease. *Cell Metabolism*, 33(12), pp.2329–2354. doi:<https://doi.org/10.1016/j.cmet.2021.09.010>.
- Hill, C.M., Laeger, T., Dehner, M.V., Albarado, D.C., Clarke, B., Wanders, D., Burke, S.J., J. Jason Collier, Qualls-Creekmore, E., Solon-Biet, S.M., Simpson, S.J., Berthoud, H., Münzberg, H. and Morrison, C.D. (2019). FGF21 Signals Protein Status to the Brain and Adaptively Regulates Food Choice and Metabolism. *Cell*

Reports, [online] 27(10), pp.2934-2947.e3.
doi:<https://doi.org/10.1016/j.celrep.2019.05.022>.

- Hossain, A., Yamaguchi, F., Matsuo, T., Tsukamoto, I., Toyoda, Y., Ogawa, M., Nagata, Y. and Tokuda, M. (2015). Rare sugar d-allulose: Potential role and therapeutic monitoring in maintaining obesity and type 2 diabetes mellitus. *Pharmacology & Therapeutics*, [online] 155, pp.49–59.
doi:<https://doi.org/10.1016/j.pharmthera.2015.08.004>.
- Inoue, M., Takaaki Ohtake, Wataru Motomura, Takahashi, N., Yayoi Hosoki, Miyoshi, S., Suzuki, Y., Saito, H., Yutaka Kohgo and Okumura, T. (2005). Increased expression of PPAR γ in high fat diet-induced liver steatosis in mice. *Biochemical and Biophysical Research Communications*, [online] 336(1), pp.215–222.
doi:<https://doi.org/10.1016/j.bbrc.2005.08.070>.
- Itoh, K., Mizuno, S., Hama, S., Oshima, W., Kawamata, M., Hossain, A., Ishihara, Y. and Tokuda, M. (2015). Beneficial Effects of Supplementation of the Rare Sugar ‘D-allulose’ Against Hepatic Steatosis and Severe Obesity in *Lep^{ob}/Lep^{ob}* Mice. *Journal of Food Science*, [online] 80(7). doi:<https://doi.org/10.1111/1750-3841.12908>.
- Ja Hyun Koo and Chang Yeob Han (2021). Signaling Nodes Associated with Endoplasmic Reticulum Stress during NAFLD Progression. *Biomolecules*, [online] 11(2), pp.242–242. doi:<https://doi.org/10.3390/biom11020242>.
- João Paulo Camporez, Jornayvaz, F.R., Petersen, M.C., Pesta, D., Guigni, B.A., Serr, J., Zhang, D., Kahn, M., Samuel, V.T., Jurczak, M.J. and Shulman, G.I. (2013). Cellular Mechanisms by Which FGF21 Improves Insulin Sensitivity in Male Mice. *Endocrinology*, [online] 154(9), pp.3099–3109. doi:<https://doi.org/10.1210/en.2013-1191>.
- Kasper and Serlie, M.J. (2017). Fructose Consumption, Lipogenesis, and Non-Alcoholic Fatty Liver Disease. *Nutrients*, [online] 9(9), pp.981–981.
doi:<https://doi.org/10.3390/nu9090981>.

- Kilkenney, D.M. and Rocheleau, J.V. (2016). The FGF21 Receptor Signaling Complex. *Vitamins and hormones*, [online] pp.17–58. doi:<https://doi.org/10.1016/bs.vh.2016.02.008>.
- Kohli, R., Kirby, M., Xanthakos, S.A., Softic, S., Feldstein, A.E., Saxena, V., Tang, P.H., Miles, L., Miles, M.V., Balistreri, W.F., Woods, S.C. and Seeley, R.J. (2010). High-fructose, medium chain trans fat diet induces liver fibrosis and elevates plasma coenzyme Q9 in a novel murine model of obesity and nonalcoholic steatohepatitis. *Hepatology*, [online] 52(3), pp.934–944. doi:<https://doi.org/10.1002/hep.23797>.
- Koliaki, C., Kokkinos, A., Tentolouris, N. and Katsilambros, N. (2010). The Effect of Ingested Macronutrients on Postprandial Ghrelin Response: A Critical Review of Existing Literature Data. *International Journal of Peptides*, [online] 2010, pp.1–9. doi:<https://doi.org/10.1155/2010/710852>.
- Kook Hwan Kim, Seong Hun Kim, Min, Y., Yang, H.-W., Lee, J. and Lee, M.-S. (2013). Acute Exercise Induces FGF21 Expression in Mice and in Healthy Humans. *PLOS ONE*, [online] 8(5), pp.e63517–e63517. doi:<https://doi.org/10.1371/journal.pone.0063517>.
- Kurosu, H., Choi, M., Ogawa, Y., Addie Smith Dickson, Goetz, R., Eliseenkova, A.V., Mohammadi, M., Rosenblatt, K.P., Kliewer, S.A. and Makoto Kuro-o (2007). Tissue-specific Expression of β Klotho and Fibroblast Growth Factor (FGF) Receptor Isoforms Determines Metabolic Activity of FGF19 and FGF21. *Journal of Biological Chemistry*, [online] 282(37), pp.26687–26695. doi:<https://doi.org/10.1074/jbc.m704165200>.
- Lakhani, I., Gong, M., Wong, W.T., Bazoukis, G., Lampropoulos, K., Wong, S.H., Wu, W.K.K., Wong, M.C.S., Ong, K.-L., Liu, T. and Tse, G. (2018). Fibroblast growth factor 21 in cardio-metabolic disorders: a systematic review and meta-analysis. *Metabolism*, 83, pp.11–17. doi:<https://doi.org/10.1016/j.metabol.2018.01.017>.
- Lee, D., Han, Y., Kwon, E. and Choi, M. (2020). d-allulose Ameliorates Metabolic Dysfunction in C57BL/KsJ-db/db Mice. *Molecules*, [online] 25(16), pp.3656–3656. doi:<https://doi.org/10.3390/molecules25163656>.

- Lehtonen, J.M., Forsström, S., Bottani, E., Viscomi, C., Baris, O.R., Isoniemi, H., Höckerstedt, K., Österlund, P., Hurme, M., Jylhävä, J., Leppä, S., Markkula, R., Heliö, T., Mombelli, G., Uusimaa, J., Laaksonen, R., Laaksovirta, H., Auranen, M., Zeviani, M. and Smeitink, J. (2016). FGF21 is a biomarker for mitochondrial translation and mtDNA maintenance disorders. *Neurology*, [online] 87(22), pp.2290–2299. doi:<https://doi.org/10.1212/WNL.0000000000003374>.
- Lewis, J., Monnier, C., Marshall, H.J., Fowler, M.J., Green, R., Cooper, S., Aristeidis Chiotellis, Lockett, J., Perkins, A.C., Tamer Coşkun, Adams, A.C., Samms, R.J., Ebling, F.J. and Kostas Tsintzas (2020). Whole-body and adipose tissue-specific mechanisms underlying the metabolic effects of fibroblast growth factor 21 in the Siberian hamster. *Molecular Metabolism*, [online] 31, pp.45–54. doi:<https://doi.org/10.1016/j.molmet.2019.10.009>.
- Li, Y., Li, S., Qiu, Y., Zhou, M., Chen, M., Hu, Y., Hong, S., Jiang, L. and Guo, Y. (2022). Circulating FGF21 and GDF15 as Biomarkers for Screening, Diagnosis, and Severity Assessment of Primary Mitochondrial Disorders in Children. *Frontiers in Pediatrics*, 10. doi:<https://doi.org/10.3389/fped.2022.851534>.
- Liang, Q., Zhong, L., Zhang, J., Wang, Y., Bornstein, S.R., Triggle, C.R., Ding, H., Karen S.L. Lam and Xu, A. (2014). FGF21 Maintains Glucose Homeostasis by Mediating the Cross Talk Between Liver and Brain During Prolonged Fasting. *Diabetes*, [online] 63(12), pp.4064–4075. doi:<https://doi.org/10.2337/db14-0541>.
- Livak, K.J. and Schmittgen, T.D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods*, [online] 25(4), pp.402–408. doi:<https://doi.org/10.1006/meth.2001.1262>.
- Lonzetti, C., Larissa Pereira Santos, Lopes, G., Márcia Barbosa Águila and Carlos Alberto Mandarim-de-Lacerda (2017). Eicosapentaenoic acid (EPA) vs. Docosahexaenoic acid (DHA): Effects in epididymal white adipose tissue of mice fed a high-fructose diet. *Prostaglandins Leukotrienes and Essential Fatty Acids*, [online] 123, pp.14–24. doi:<https://doi.org/10.1016/j.plefa.2017.07.004>.
- Luísa, A., Relat, J., Hondares, E., Pérez-Martí, A., Ribas, F., Francesc Villarroya, Marrero, P.F. and Haro, D. (2013). FGF21 mediates the lipid metabolism response to

amino acid starvation. *Journal of Lipid Research*, [online] 54(7), pp.1786–1797. doi:<https://doi.org/10.1194/jlr.m033415>.

- Lundåsen, T., Hunt, M.C., Nilsson, L.-M., Sanyal, S., Angelin, B., Stefan E.H. Alexson and Mats Rudling (2007). PPAR α is a key regulator of hepatic FGF21. *Biochemical and Biophysical Research Communications*, [online] 360(2), pp.437–440. doi:<https://doi.org/10.1016/j.bbrc.2007.06.068>.
- Markan, K.R., Naber, M.C., Ameka, M.K., Anderegg, M.D., Mangelsdorf, D.J., Kliewer, S.A., Mohammadi, M. and Potthoff, M.J. (2014). Circulating FGF21 Is Liver Derived and Enhances Glucose Uptake During Refeeding and Overfeeding. *Diabetes*, 63(12), pp.4057–4063. doi:<https://doi.org/10.2337/db14-0595>.
- Martín Alcalá, María Calderón-Domínguez, Bustos, E., Ramos, P., Casals, N., Serra, D. and Herrero, L. (2017). Increased inflammation, oxidative stress and mitochondrial respiration in brown adipose tissue from obese mice. *Scientific Reports*, [online] 7(1). doi:<https://doi.org/10.1038/s41598-017-16463-6>.
- Messier, C., Whately, K., Liang, J., Du, L. and Puissant, D. (2007). The effects of a high-fat, high-fructose, and combination diet on learning, weight, and glucose regulation in C57BL/6 mice. *Behavioural Brain Research*, 178(1), pp.139–145. doi:<https://doi.org/10.1016/j.bbr.2006.12.011>.
- Pan, Q., Lin, S., Li, Y., Liu, L., Li, X., Gao, X., Yan, J., Gu, B., Chen, X., Li, W., Tang, X., Chen, C. and Guo, L. (2021). A novel GLP-1 and FGF21 dual agonist has therapeutic potential for diabetes and non-alcoholic steatohepatitis. *EBioMedicine*, 63(63), p.103202. doi:<https://doi.org/10.1016/j.ebiom.2020.103202>.
- Pereira, L., Figueredo, G.A., Natália Oliveira Bertolini, Ceccato, M., Jessica Rodrigues Pereira, Christine, A. and Rostom, A. (2012). Dietary restriction, caloric value and the accumulation of hepatic fat. *Lipids in Health and Disease*, [online] 11(1), pp.2–2. doi:<https://doi.org/10.1186/1476-511x-11-2>.
- Petya Hadzhibozheva, Liliya Pashova-Stoyanova, Zhivka Tsokeva, María Ganeva, Krasimira Nancheva, G. Ilieva, Velcho Nanchev, A. Tolekova and Georgiev, T. (2023). Appetite–regulating hormones in rats with fructose-induced metabolic

changes. *Фармація*, [online] 70(1), pp.1–7.
doi:<https://doi.org/10.3897/pharmacia.70.e87712>.

- Picó, C., Catalina Amadora Pomar and Rodríguez, A. (2021). Leptin as a key regulator of the adipose organ. *Reviews in Endocrine and Metabolic Disorders*, [online] 23(1), pp.13–30. doi:<https://doi.org/10.1007/s11154-021-09687-5>.
- Prida, E., Álvarez-Delgado, S., Pérez-Lois, R., Mateo Soto-Tielas, Estany-Gestal, A., Johan Fernø, Seoane, L.M., Mar Quiñones and Al-Massadi, O. (2022). Liver Brain Interactions: Focus on FGF21 a Systematic Review. *International Journal of Molecular Sciences*, [online] 23(21), pp.13318–13318.
doi:<https://doi.org/10.3390/ijms232113318>.
- Rubén Nogueiras, Tschöp, M.H. and Zigman, J.M. (2008). *Central Nervous System Regulation of Energy Metabolism. Annals of the New York Academy of Sciences*, [online] 1126(1), pp.14–19. doi:<https://doi.org/10.1196/annals.1433.054>.
- Sabrina Azevedo Machado, Pasquarelli-do-Nascimento, G., Santos, D., Gabriel Ribeiro Farias, Santos, Luana Borges Baptista and Kelly Grace Magalhães (2022). Browning of the white adipose tissue regulation: new insights into nutritional and metabolic relevance in health and diseases. *Nutrition & Metabolism*, [online] 19(1). doi:<https://doi.org/10.1186/s12986-022-00694-0>.
- Sally Yu Shi, Lu, Y., Richardson, J., Min, X., Weizmann, J., Richards, W.G., Zhong Lin Wang, Zhang, Z., Zhang, J. and Li, Y. (2018). A systematic dissection of sequence elements determining β -Klotho and FGF interaction and signaling. *Scientific Reports*, [online] 8(1). doi:<https://doi.org/10.1038/s41598-018-29396-5>.
- Sang Hoon Kim, Su Jeong Kim, Kim, H.-J. and Sung, M. (2017). d-Psicose, a sugar substitute, suppresses body fat deposition by altering networks of inflammatory response and lipid metabolism in C57BL/6J-ob/ob mice. *Journal of Functional Foods*, [online] 28, pp.265–274. doi:<https://doi.org/10.1016/j.jff.2016.11.029>.
- Solon-Biet, S.M., Cogger, V.C., Pulpitel, T., Marika Heblinski, Wahl, D., McMahon, A.C., Warren, A., Durrant-Whyte, J., Walters, K.A., Krycer, J.R., Ponton, F., Rahul Gokarn, Wali, J.A., Ruohonen, K., Conigrave, A.D., James, D.E., Raubenheimer, D.,

Morrison, C.D., Le, D.G. and Simpson, S.J. (2016). Defining the Nutritional and Metabolic Context of FGF21 Using the Geometric Framework. *Cell Metabolism*, [online] 24(4), pp.555–565. doi:<https://doi.org/10.1016/j.cmet.2016.09.001>.

- Straub, L.G. and Wolfrum, C. (2015). FGF21, energy expenditure and weight loss – How much brown fat do you need? *Molecular Metabolism*, [online] 4(9), pp.605–609. doi:<https://doi.org/10.1016/j.molmet.2015.06.008>.
- Takashi Nomiyama, Pérez-Tilve, D., Ogawa, D., Gizard, F., Zhao, Y., Heywood, E.B., Jones, K.L., Ryuzo Kawamori, Cassis, L.A., Tschöp, M.H. and Bruemmer, D. (2007). Osteopontin mediates obesity-induced adipose tissue macrophage infiltration and insulin resistance in mice. *Journal of Clinical Investigation*, [online] 117(10), pp.2877–2888. doi:<https://doi.org/10.1172/jci31986>.
- Tezze, C., Romanello, V. and Sandri, M. (2019). FGF21 as Modulator of Metabolism in Health and Disease. [online] 10. doi:<https://doi.org/10.3389/fphys.2019.00419>.
- Tucker, W., Tucker, B., Rye, K.-A. and Ong, K.L. (2022). Fibroblast growth factor 21 in heart failure. *Heart Failure Reviews*. doi:<https://doi.org/10.1007/s10741-022-10268-0>.
- Vega, R.B., Huss, J.M. and Kelly, D.P. (2000). The Coactivator PGC-1 Cooperates with Peroxisome Proliferator-Activated Receptor α in Transcriptional Control of Nuclear Genes Encoding Mitochondrial Fatty Acid Oxidation Enzymes. *Molecular and Cellular Biology*, [online] 20(5), pp.1868–1876. doi:<https://doi.org/10.1128/mcb.20.5.1868-1876.2000>.
- Yamazaki, T., Okawa, S. and Mayumi Takahashi (2016). The effects on weight loss and gene expression in adipose and hepatic tissues of very-low carbohydrate and low-fat isoenergetic diets in diet-induced obese mice. *Nutrition & Metabolism*, [online] 13(1). doi:<https://doi.org/10.1186/s12986-016-0139-1>.
- Yano, K., Yamaguchi, K., Seko, Y., Shinya Okishio, Hiroshi Ishiba, Nozomi Tochiki, Takahashi, A., Kataoka, S., Okuda, K., Liu, Y., Fujii, H., Atsushi Umemura, Moriguchi, M., Takeshi Okanoue and Itoh, Y. (2022). Hepatocyte-specific fibroblast growth factor 21 overexpression ameliorates high-fat diet-induced obesity and liver

steatosis in mice. *Laboratory Investigation*, [online] 102(3), pp.281–289.

doi:<https://doi.org/10.1038/s41374-021-00680-9>.

- Young Mee Chung, Lee, J., Deuk Youl Kim, Se Hee Hwang, Young Ho Hong, Seong Bo Kim, Song Jin Lee and Chi Hye Park (2012). Dietary d-Psicose Reduced Visceral Fat Mass in High-Fat Diet-Induced Obese Rats. *Journal of Food Science*, [online] 77(2). doi:<https://doi.org/10.1111/j.1750-3841.2011.02571.x>.
- Zeng, K., Tian, L., Patel, R., Shao, W., Song, Z., Liu, L., Manuel, J., Ma, X., McGilvray, I., Cummins, C.L., Weng, J. and Jin, T. (2016). Diet polyphenol curcumin stimulates hepatic Fgf21 production and restores its sensitivity in high fat diet fed male mice. *Endocrinology*, [online] pp.jc.2016.1596–jc.2016.1596. doi:<https://doi.org/10.1210/en.2016-1596>.
- Zhang, Y., Xie, Y., Berglund, E.D., Coate, K.C., He, T., Tetsuro Katafuchi, Xiao, G., Potthoff, M.J., Wei, W., Wan, Y., Yu, R.T., Evans, R.M., Kliewer, S.A. and Mangelsdorf, D.J. (2012). The starvation hormone, fibroblast growth factor-21, extends lifespan in mice. *eLife*, [online] 1. doi:<https://doi.org/10.7554/elife.00065>.

APPENDIX:

Summary of Tests of Normality

Non-Normally distributed data			
<i>Metric:</i>	<i>Diet:</i>	<i>Shapiro-Wilk:</i>	<i>Significance:</i>
ΔCT	HFDf	0.858	0.046
$\Delta\Delta CT$	HFDf	0.852	0.039
$2^{-\Delta\Delta Ct}$	HFDa	0.732	0.001
Triglyceride mg/g	HFDf	0.848	0.035
Spleen g/100g	HFDf	0.848	0.035
Liver(g)	HFDw	0.834	0.05
FGF21(ng)	HFDf	0.833	0.023

Table 1 above displays the summary of important results for the Shapiro-Wilk test conducted on data metrics from each diet. Post imputation (as described in section 2.7 above), several metrics were found to be statistically significant within the Shapiro-Wilk test, which is indicative of Non-Normally distributed data. Namely HFDf in FGF21, spleen weight, Triglyceride content, $\Delta\Delta CT$ & ΔCT metrics, HFDw is non-normally distributed for liver weight, HFDa is non-normally distributed for $2^{-\Delta\Delta Ct}$ measurements.

qPCR metrics

	LDw		HFDw		HFDf		HFDa	
	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation	Mean	Std. Deviation
ΔCT	6.53	1.38	6.56	1.49	7.23	0.21	8.30	1.47
ΔΔCT	0.0020	1.38	0.03	1.49	0.68	0.21	1.74	1.47
2^{-ΔΔCT}	0.98	0.55	0.73	0.53	0.62	0.09	0.22	0.13
FGF21 CT	33.47	1.92	31.97	2.30	32.64	2.39	34.11	1.92
PPIACT CT	26.94	2.10	25.41	2.01	25.65	2.09	25.81	2.36

	HFDf		HFDa	
	Minimum	Maximum	Minimum	Maximum
ΔCT	7	7.62	5.39	11.04
ΔΔCT	0.45	1.06	-1.17	4.48
2^{-ΔΔCT}	0.48	0.73	0	0.32
FGF21 CT	28.39	36.28	31.22	36.89
PPIACT CT	20.78	29.01	21.99	30.12

The table above contains descriptive qPCR statistics for each of the diets analysed during the course of this study. Each metric measured when comparing HFDw, LDw & HFDf diets were not statistically significant.

However, ΔCT, ΔΔCT, & 2^{-ΔΔCT} metrics are statistically significant when HFDa is compared to HFDw (P=0.006, P=0.006, P=0.01) & HFDf respectively (P=0.009, P=0.009, P<0.001). Both sets were tested with Mann-Whitney U test as data is non-normally distributed. This here is clearly seen with a 2^{-ΔΔCT} of 0.2182 (on average) when compared to the scores of 0.6158 & 0.7256 of HFDf and HFDw. This is further compounded by the glaring differences in ΔΔCT scores, of 1.74 for HFDa, 0.675 for HFDf & 0.0289 for HFDw.

It must also be noted that HFDf is the tightest grouped of each diet here, with a standard deviation approximately ~7x less than either HFDa or HFDw. The PPIA CT average metric here serve to describe the consistency of sample preservation across RNA extraction, cDNA generation & qPCR analysis.

LDw Correlations:

LDw		
<i>Correlation:</i>	<i>Significance (P value):</i>	<i>Pearson Coefficient:</i>
Leptin/ EAT	0.002	0.854
Leptin/ Total AT	0.002	0.842
Leptin/ OGTT 120 mins	0.046	0.64
Ghrelin/ Brown AT	0.003	-0.825

Fold Change Calculations:

Diet	$2^{-\Delta\Delta Ct}$	Formula	Fold Change
LDw	0.98	$\text{LOG}(0.98/0.98, \{2\})$	0
HFDw	0.73	$\text{LOG}(0.73/0.98, \{2\})$	-0.42
HFDf	0.63	$\text{LOG}(0.62/0.73, \{2\})$	-0.24
HFDa	0.22	$\text{LOG}(0.22/0.73, \{2\})$	-1.73

Fold change is calculated in respect to individual controls: LDw acts as the control for LDw

& HFDw, HFDw is the control for HFDf & HFDa.

RNA -Extract Qualitative & Quantitative assessment

<i>RNA Extraction summary - concentration & OD ratio *</i>												
Mouse ID	73	71	72	74	40	37	39	38	79	80		
	3.067	2.21	1.654	1.472	2.081	2.063	1.55	N/A	N/A	N/A		
	Conc. (ng/μL)	9.748	17.007	7.287	4.478	30.418	8.369	13.042	N/A	N/A	N/A	
LDw												
Mouse ID	35	36	77	76	78	75	75					
	2.497	3.763	2.015	1.863	1.964	3.85	2.174					
	Conc. (ng/μL)	15.294	9.439	104.937	18.537	18.478	6.527	28.236				
HFDw												
Mouse ID	7	8	57	9	10	57	58	10	11	12	53	55
	4.154	2.076	2.162	2.04	2.024	2.138	2.189	1.976	2.333	2.294	N/A	N/A
	Conc. (ng/μL)	11.438	51.091	454.246	30.249	14.371	108.837	35.374	14.286	7.713	26.474	N/A
HFDf												
Mouse ID	3	5	4	45	1	2	6	45	46	43	44	
	28.8	1.718	10.457	2.417	2.099	2.582	2.283	1.694	2.023	N/A	N/A	
	Conc. (ng/μL)	6.08	11.822	15.453	4.898	12.582	19.911	24.504	10.199	21.974	N/A	N/A
HFDa												

**(sorted in chronological order of extraction)*

Dietary intake – Detailed breakdown

The four experimental groups with 14 weeks of treatment (lean diet (LDw) (n=10), high fat diet (HFD) + water (HFDw) (n=10); HFD + Fructose (HFDf) (n=12); HFD + Allulose (HFDa) (n=12)) were not kept in metabolic individual cages but housed in groups of 4 and 2 animals, so the diet and drinking measures are by cage and treatment.

Considering the difference in the energy content (in Kcal) for the Lean and HFD diets, it is possible to see that LDw had caloric ingestion 18% smaller than the HFDw treatment ($p < 0.05$) (Table S1). This higher caloric intake is reflected in the weight gain of both groups, as shown elsewhere.

Table S1. Average diet daily intake (g) and average diet daily energy intake (kcal), during 14 weeks of treatment

Diet	LDw	HFDw	HFDf	HFDa
Average daily intake/ animal (g)	3.2 ± 0.5	3.2 ± 0.5	3.3 ± 0.6	3.4 ± 0.8
Average daily energy intake/animal (Kcal)	12.5 ± 1.9*	15.3 ± 2.2	15.5 ± 2.9	15.8 ± 3.8

(*) Express statistical difference between LDw and HFDw groups ($p < 0.05$)

(a, b) Different letters express statistical differences among HFDw, HFDf and HFDa (Oneway Anova, followed by Sidak test, $p < 0.05$)

The lean diet contains a 10% low-fat diet (Lean; #D12450B; Research Diets, USA; % by values of energy) and it has 3.85 Kcal/g.

The high-fat diet (Research Diets, USA (3) 45% fat (HFD-CAS; #D12451) has 4.72 Kcal/g.

The HFD has 18% more calories per g than the Lean diet.

Mice were administered the sweeteners (dissolved in autoclaved drinking water) Allulose (C&J, 4%), Fructose (Sigma, 4%), or water during the 14 weeks study duration.

As shown in Table S2, the average daily drinking water intake (mL) for the four groups was similar (no statistical difference), showing a good acceptance of the water and sweetener solutions and a similar drinking pattern.

Due to the caloric values of the sweeteners added to the water, the energy intake among the three experimental groups under HFD was significantly different. The higher caloric intake for the HFDf group reflected on a higher weight gain for this group.

However, the HFDa group showed a smaller weight gain despite a higher energy intake compared to the HFDw groups.

Table S2. Average daily drinking water intake (mL) and average daily energy intake (kcal), during 14 weeks of treatment

Drinking Water	LDw	HFDw	HFDf	HFDa
Average daily intake/ animal (mL)	4.4 ± 0.7	4.5 ± 1.3	4.9 ± 0.9	4.8 ± 1.2
Average daily energy intake/animal (Kcal)	0	0 ^a	0.79 ± 0.15 ^b	0.08 ± 0.02 ^c

(*) Express statistical difference between LDw and HFDw groups (p<0.05)

(a, b) Different letters express statistical differences among HFDw, HFDf and HFDa (Oneway Anova, followed by Sidak test, p <0.05)

Fructose has 4 kcal/g, so the 4% solution has 0.16 kcal/mL. Allulose has 0.4 Kcal per gram, so the 4% solution has 0.016 kcal/mL.

Dietary Intervention – Detailed breakdown

The study that generated the analytical data used in this thesis was performed by Dr. Fabiana A Hoffmann Sarda and collaborators, under the supervision of Dr. Catherine Stanton and Dr. John Cryan. It was done during her Marie Skłodowska-Curie Action (MSCA) – Horizon 2020, Grant agreement No 754535, in APC Microbiome, UCC, Cork Ireland.

- Study design

C57BL/6 J (Envigo) adult male mice (4 or 2 animals) were housed under a 12 h light-dark cycle, under experimental LCS in drinking water and diet. They were split into two cohorts of 22 animals each. Each cohort has 4 experimental groups with 14 weeks of treatment: lean diet (LDw) (n=10), high fat diet (HFD) + water (HFDw) (n=10); HFD + Fructose (HFDf) (n=12); HFD + Allulose (HFDa) (n=12).

Weight was monitored weekly, and food intake and drinking water were monitored 3 times a week. All the animals will be euthanized at the end of the study.

- Ethical approval

The *in vivo* experiments were approved by the University College Cork Animal Experimentation Ethics Committee (Project AE 19130- P039), were licensed under the European Directive 2010/63/EU, and comply with the ARRIVE guidelines. Thirty-four C57BL/6J 5-week-old male mice were purchased commercially (Envigo; UK) and were housed 4 or 2 per cage on a 12 hr light/dark cycle. The mice had *ad libitum* access to food and water throughout the study unless otherwise stated. During the initial 2 weeks of the acclimatization period, mice were provided with a diet containing a 10% low-fat diet (Lean; #D12450B; Research Diets, USA; % by values of energy). Subsequently, weight-matched mice received a high-fat diet (Research Diets, USA (3) 45% fat (HFD-CAS; #D12451) and drinking water, or sweeteners, and the control group remained receiving the Lean diet (#D12450B; Research Diets, USA).

The Lean diet (D12450H) has 3.85 Kcal/g, and the HFD diet (D12451) has 4.72 Kcal/g.

The HFD has 18% more calories per g than the Lean diet.

- Sweeteners Administration

Mice were administered the sweeteners (dissolved in autoclaved drinking water) Allulose (C&J, 4%), Fructose (Sigma, 4%), or water during the 14 weeks study duration. Fructose has 4 kcal/g, so the 4% solution has 0.16 kcal/mL. Allulose has 0.4 Kcal per gram, so the 4% solution has 0.016 kcal/mL.

Duration of treatment was chosen based on previous studies in rodents showing and neurochemical effects following 8 to 12 weeks of treatment with sweeteners (Burokas et al, 2017, van de Wouw et al, 2018).

- Weight assessment

Body weight was measured weekly, as well as the food intake per cage, drinking water and Sweeteners solutions intake were measured twice a week.

Mice fasted for 10 h, commencing at 22.00 in the dark phase. Mice were euthanized by cervical dislocation and blood samples and tissues were collected. Tissues weight and/or length were recorded, and the samples were stored at -80°C for subsequent analysis.

- Metabolic assessment = glucose homeostasis was assessed by OGTT test with tail bleeding (Nagy and Einwallner, 2018)

Mice were fasted overnight on clean bedding, with free access to water drinking solutions. Early in the morning, animal weight was recorded to calculate the volume of administered glucose. D-Glucose was administered by oral gavage in sterile 0.8% NaCl at 1.5 g/kg of body weight (20% glucose solution, 200-250 μL per mouse). After the gavage, animals are single housed in clean cages until the end of the test. Glucose levels are measured in tail vein blood using a glucometer (Bayer, UK) before (0 min) and 15, 30, 60, 90 and 120 min after the gavage. At each time point, glucose is measured in duplicate (4 μL of blood in total). 100 μL of blood (1.5 capillaries) are collected at 0 min time point to measure fasting insulin and other hormones. 50 μL of blood (3/4 capillary) are collected at each time point to measure the insulin response.

- Glucose, insulin, and triacylglycerol levels

Mouse Insulin ELISA Kits, Ultra-Sensitive Assay (Crystal Chem, USA) was used to determine the plasma insulin level.

The weights of liver were recorded, and the samples were homogenized in 1,5 ml of %NP40/ddH₂O solution. After two repeated steps of heating (80°C – 100°C for 3–5 min) and cool down (15 min at RT), all the samples were centrifuged at maximum speed. The

supernatant, containing the lipids, was collected and diluted 1:10. Triacylglycerol (TAG) level in the liver extract was measured using Triglyceride Quantification Assay Kit (Abcam, UK).

- FGF-21, Leptin, Ghrelin, GLP-1

The carotid blood was collected in EDTA tube (final concentration; 50 IU ml⁻¹), aprotinin (final concentration; 500 kIU ml⁻¹), and DPP-IV inhibitor vildagliptin (final concentration; 10 µM). Plasma was collected after centrifugation (3000 × g, 10 min at 4 °C) and stored at -80 °C until assay. GLP-1, FGF-21, Leptin and Ghrelin, levels were measured using U-PLEX Metabolic Assays (MSD).

References

Nagy, C. and Einwallner, E. (2018) 'Study of In Vivo Glucose Metabolism in High-fat Diet-fed Mice Using Oral Glucose Tolerance Test (OGTT) and Insulin Tolerance Test (ITT)', *Journal of Visualized Experiments : JoVE*, (131), p. 56672. Available at: <https://doi.org/10.3791/56672>.

Burokas A, Arboleya S, Moloney RD, Peterson VL, Murphy K, Clarke G, et al. Targeting the Microbiota-Gut-Brain Axis: Prebiotics Have Anxiolytic and Antidepressant-like Effects and Reverse the Impact of Chronic Stress in Mice. *Biol Psychiatry*. 2017 Oct 1;82(7):472–87.

van de Wouw M, Boehme M, Lyte JM, Wiley N, Strain C, O'Sullivan O, et al. Short-chain fatty acids: microbial metabolites that alleviate stress-induced brain-gut axis alterations. *J Physiol*. 2018 Oct 15;596(20):4923–44.