Diploma thesis

The specific role of Fibroblast Growth Factor 21 concerning alcohol and sugar intake and its changes after a meal tolerance test.

FGF21: a potent pharmacological target to prevent alcohol dependence and diverse features of the metabolic syndrome?

submitted by

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Graz, 05 May 2020

Affidavit

I hereby declare that the following diploma thesis has been written only by the undersigned and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Graz, 05 May 2020 *Lukas Kofler eh*

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Abstract

Introduction

Obesity, diabetes and other features of the metabolic syndrome, as well as alcohol related diseases are common health problems. The Fibroblast Growth Factor (FGF) 21 has been previously shown to play a fundamental role in metabolic disorders. The aim of the study was to investigate the effect of alcohol and variant nutrient solutions on plasma FGF21 levels in healthy subjects.

Materials and Methods

In this interventional study four oral solutions were trialled for their effects on FGF21 levels: alcohol, glucose, fructose and a mixed caloric drink (MCD). Blood samples were taken before and up to four hours after the ingestion. FGF21 concentrations were measured by a human FGF21-ELISA-kit. Altogether, 63 healthy and diabetic subjects were included. All cohorts were selected from the University Hospital of Graz.

Results

FGF21 concentrations increased rapidly after alcohol and fructose ingestion with a mean 10.3-fold and 2.7-fold increase at two hours compared to baseline ($p=0.001$ and $p=0.005$, respectively). In contrast, FGF21 did not increase in the first two hours following ingestion of a glucose load and decreased 2.2-fold two hours after ingestion of a MCD compared to baseline ($p=0.007$) in healthy subjects. However, FGF21 concentrations did not change after ingestion of the MCD in the diabetic group. Moreover, the comparison of the baseline FGF21 levels between the diabetic subjects and the healthy group showed that those in the diabetic group were 4.5-fold higher ($p\leq0.001$).

Discussion

Alcohol and fructose ingestion rapidly and robustly elevate human plasma FGF21 levels with a peak at two hours, while ingestion of a MCD suppresses them. Glucose, on the other hand, does not influence FGF21 concentrations significantly. Those findings indicate that FGF21 is affected by various nutrient solutions in a different manner. However, the question if FGF21 could be a potent pharmacological target to prevent diverse metabolic disorders, might be answered soon.

Zusammenfassung

Einleitung

Adipositas, Diabetes und andere Teilaspekte des Metabolischen Syndroms, sowie alkoholbedingte Erkrankungen sind allgegenwärtige Gesundheitsprobleme. Die fundamentale Rolle des Fibroblasten-Wachstumsfaktors (FGF) 21 bezüglich dieser metabolischen Störungen wurde in Vergangenheit bereits aufgezeigt. Das Ziel dieser Studie war es, den Effekt von Alkohol und verschiedenen Nährstofflösungen auf den Plasma-FGF21-Spiegel von gesunden Probandinnen und Probanden zu erforschen.

Material und Methoden

In dieser interventionellen Studie wurden vier verschiedene perorale Lösungen bezüglich deren Effekt auf FGF21-Spiegel untersucht: Alkohol, Glukose, Fruktose und eine gemischt kalorische Trinknahrung (GKT). Blutproben wurden vor und bis zu vier Stunden nach dem Konsum abgenommen. FGF21-Konzentrationen wurden mit einem humanen FGF21-ELISA-Kit gemessen. Insgesamt wurden 63 gesunde Probandinnen und Probanden bzw. Diabetikerinnen und Diabetiker inkludiert. Alle Kohorten wurden am Universitätsklinikum Graz rekrutiert.

Ergebnisse

FGF21-Konzentrationen stiegen akut nach der Alkohol- und Fruktoseeinnahme mit einem durchschnittlich 10.3- bzw. 2.7-fachen Anstieg nach zwei Stunden verglichen zum Startwert ($p=0001$ bzw. $p=0.005$). Nach Glukoseeinnahme stieg FGF21 in den ersten zwei Stunden hingegen nicht signifikant an. Im Gegensatz dazu sank FGF21 2.2-fach zwei Stunden nach Einnahme einer GKT, verglichen zum Startwert ($p=0.007$) in gesunden Probandinnen und Probanden. Die Konzentrationen veränderten sich jedoch nicht bei der diabetischen Gruppe nach der Einnahme der GKT. Darüber hinaus zeigte sich im Startwertvergleich der FGF21-Spiegel zwischen Diabetikerinnen bzw. Diabetikern und der gesunden Gruppe, dass jene in der diabetischen Kohorte 4.5-fach höher sind ($p\leq0.001$).

Diskussion

Alkohol- und Fruktoseaufnahme führen zu einer schnellen und robusten Erhöhung von Plasma-FGF21-Spiegeln im Menschen mit einem Maximum bei zwei Stunden, während die Einnahme einer GKT zu einer Suppression führt. Glukose hingegen beeinflusst FGF21 Spiegel nicht signifikant. Diese Ergebnisse weisen darauf hin, dass FGF21 von verschiedenen Nährstofflösungen in unterschiedlicher Weise beeinflusst wird. Wie auch immer, die Frage, ob FGF21 ein potentes pharmakologisches Ziel sein könnte, um verschiedene metabolische Störungen zu vermeiden, wird möglicherweise bald beantwortet werden.

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Abbreviations & glossary

A

ACE inhibitors	*Angiotensin-converting enzyme inhibitors*
adj.	*Adjusted*
α-Klotho	*Alpha-Klotho*
ALT	*Alanine transaminase*
AP	*Alkaline phosphatase*
AST	*Aspartate transaminase*
AT1 receptor blockers	*Angiotensin II type 1 receptor blockers*

B

Bili	*Bilirubin*
β-Klotho	*Beta-Klotho = KLB*
BLU9931	*FGF-receptor 4 inhibitor*
BMI	*Body mass index*

C

CAGE-test	*Screening test for problem drinking and potential alcohol problems*
CHE	*Cholinesterase*
ChREBP	*Carbohydrate response element binding protein*
CYP7A1	*Cytochrome P450 7A1*

E

ELISA	*Enzyme-linked Immunosorbent Assay*

F

FGF	*Fibroblast growth factor*
FGFR	*Fibroblast growth factor receptor*

G

GGT	*Gamma glutamyltransferase*

H

HbA1c	*Glycated haemoglobin*

HCC	*Hepatocellular carcinoma*
HDL(-C)	*High-density lipoprotein (cholesterol)*
HRP	*Horseradish peroxidase*
K	
KLB	*Beta-Klotho = β-Klotho*
L	
LDL(-C)	*Low-density lipoprotein (cholesterol)*
LY2405319	*FGF21 analogue*
M	
MCD	*Mixed caloric drink*
mRNA	*Messenger ribonucleic acid*
MTT	*Meal tolerance test*
O	
oGTT	*Oral glucose tolerance test*
OECD	*Organisation for Economic Co-operation and Development*
P	
PPARα	*Peroxisome proliferator-activated receptor alpha*
PPARγ	*Peroxisome proliferator-activated receptor gamma*
R	
R1Mab	*Monoclonal anti-FGF-receptor 1 antibody*
rpm	*Rounds per minute*
rs838133	*FGF21 gene locus*
T	
TNFα	*Tumor necrosis factor alpha*
Z	
ZMF	*Zentrum für Medizinische Grundlagenforschung*

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1 Introduction

1.1 Scientific background of the study

Obesity is a major problem in many countries in the world. Notably, the prevalence of obesity in Austria was 16,0% among men and 13,4% among women in 2014 (the nearest year of recording) according to the OECD (Organisation for Economic Co-operation and Development) (1). It is well established that obesity is related to a high ingestion of drinks and meals with high amounts of both fructose and glucose. As a consequence, obesity can also result in insulin resistance and diabetes mellitus type 2. In this context, overweight is also a main factor for the definition of the metabolic syndrome. However, not only healthy eating is fundamental for the prevention of diverse metabolic diseases, also stopping excessive alcohol consumption leading to both alcohol addiction and, among others, to liver diseases is needed to stay healthy (2). The World Health Organization found out, that in 2004 globally more than 30 million deaths were caused by chronic diseases and conditions as a result of pathologic alcohol consumption (3). Perspicuous, because alcohol is not only associated to liver cirrhosis but also to diverse malignant tumor or cardiovascular diseases or accidents, to list a few (4).

Therefore, it is an unmet clinical need to find potential pharmacological therapies to prevent the desire for alcohol or simple sugars and hence the development of obesity, diabetes and other features of the metabolic syndrome, as well as alcohol related diseases.

One possible candidate could be the Fibroblast Growth Factor 21 – short: FGF21. It has been shown that this metabolic hormone has a strong immediate impact on some blood parameters in patients with obesity or diabetes mellitus type 2 and hence patients with glucose intolerance or insulin resistance imposed by elevated blood levels. In addition, correlations between levels of FGF21 and fasting blood glucose concentrations, levels of glycated haemoglobin (Hb1Ac) and blood glucose concentrations after an oral glucose tolerance test (oGTT) have been reported. Levels of FGF21 are also an independent marker for the presence of metabolic syndrome in obesity in adults (5).

In regard to glucose and lipid metabolism, FGF21 has many beneficial effects (6). In this context, FGF21 gene transcription in humans is increased through fasting and feeding signals (7). Not only glucose and xylitol (feeding signals), but also glucagon and free fatty acids (fasting state) induce FGF21 expression through different transcription factors; while the carbohydrate response element binding protein (ChREBP) is activated by carbohydrates, the peroxisome proliferator-activated receptor alpha (PPARα) is activated by free fatty acids (7). An active peroxisome proliferator-activated receptor gamma (PPARγ) is essential for FGF21 effects after feeding (8).

Additionally, FGF21 regulates alcohol consumption through the central nervous system in mice, indicating that FGF21 can cross the blood-brain-barrier (9). FGF21 administration, for instance, can dramatically decrease preference for alcohol in rodents (9). Moreover, water drinking behaviour after both alcohol consumption or a ketogenic diet in mice is regulated by FGF21 (10).

In this study we aimed to assess plasma levels of FGF21 after diverse oral challenges. Because FGF21 is known to play an essential role in many different metabolic processes, we wanted to investigate the impact of alcohol and sugars concerning plasma levels of FGF21 in humans. In the course of this study, we also wanted to achieve an understanding of the effect of a mixed caloric drink on FGF21 levels.

1.2 Physiology of FGF21

1.2.1 FGF expression and their (co)receptors

The Fibroblast Growth Factor 21 is one of 22 members of the FGF family in mice and humans (11). Most of the members bind directly to the FGF-receptors on the cell surfaces and need heparan sulfate for stabilization. Heparan sulfate is located in the extracellular matrix. Therefore, in large part FGFs operate locally and affect cell growth and differentiation through autocrine and paracrine signalling, because the newly formed complex cannot enter the circulation (12, 13).

In contrast, FGF21 and other FGF19 subfamily members, including FGF15/19 and FGF23, do not bind heparan sulfate that strongly and act systemically through endocrine signalling. Instead, they need other coreceptors to activate FGF signalling and to bind strongly to the FGF-receptors. FGF21 and FGF15/19 need the transmembrane protein beta-Klotho (β-Klotho = KLB), FGF23 needs the protein alpha-Klotho (α-Klotho) (14). Four different FGF-receptors lead to specific effects after activation. Beyond that, FGF-receptor 1, 2 and 3 can be subdivided into epithelial isoforms with the suffix b or mesenchymal isoforms with the suffix c (15). FGF19 and FGF21 can activate FGF signalling in cells which express both, β-Klotho and the FGF-receptor isoforms 1c, 2c or 3c. FGF19 can also bind cells with a combination of FGF-receptor 4 and β-Klotho, but FGF21 is not able to signal through this complex (16).

So, the tissue-specific metabolic effects of FGF21 can be explained by the expression of β-Klotho in combination with specific FGF-receptor isoforms (16). In mice β-Klotho is mainly expressed in the adipose tissue, the liver and the endocrine and exocrine pancreas (17-19). While fat cells express β-Klotho and FGF-receptor 1, liver cells express β-Klotho and FGF-receptor 4. Therefore, on the one hand, both, FGF19 and FGF21, can decrease blood glucose levels because they both stimulate glucose uptake in the lipocytes. On the other hand, only FGF19 can inhibit transcription of cytochrome P450 7A1 (CYP7A1) in the liver cells to regulate bile acid biosynthesis (16, 20). The interaction between FGF19 and FGF-receptor 4 in the liver also leads to cell proliferation. Hence, FGF-receptor 4 antibodies, like BLU9931, are in discussion for the therapy of hepatocellular carcinoma (HCC), because they can inhibit abnormal FGF-receptor 4 signalling after FGF19 activation (21).

Wu et al. found an antibody that can activate the FGF-receptor 1 to act like FGF21 to fight diabetes in obese mice. The monoclonal anti-FGF-receptor 1 antibody (R1MAb) is able to decrease elevated blood glucose levels acutely but also chronically and it decreases blood insulin levels, blood lipid levels and also steatosis of the liver (22).

Furthermore, recent studies have shown that FGF21 may act on multiple brain regions to control simple sugar and alcohol consumption (23).

1.2.2 FGF21: systemic and tissue-specific effects

As mentioned before, FGF21 binds heparan sulfate with low affinity. This weak binding is important for its function as an endocrine factor, because it prevents FGF21 from being captured in the extracellular matrix (24).

There are differences between the systemic and the tissue-specific effects of FGF21 in mice and humans. At lower levels, FGF21 acts physiologically in specific organ systems and tissues (8). In fasting or after the consumption of a ketogenic diet, fatty acids activate the peroxisome proliferator-activated receptor α (transcription factor PPARα) in the liver and this transcription factor induces the expression of FGF21 in the promotor regions of murine and human Fgf21 genes by binding to the PPARα responsive elements (8, 25, 26). This leads to gluconeogenesis, ketogenesis, torpor and prevention of somatic growth (8). When receiving a ketogenic diet FGF21 knockout mice gain weight, they develop steatosis of the liver (disordered oxidation of fatty acids) and they have a disordered glucose control and ketogenesis (27). In the fed state, however, the peroxisome proliferator-activated receptor γ (transcription factor PPARγ) in the white adipose tissue induces FGF21 expression to increase PPARγ activity. The autocrine induction of FGF21 in white adipose tissue now fails to increase circulating levels of FGF21 (8).

Cuevas-Ramos et al. revealed that also physical activity can induce FGF21 expression. Their 60 young female subjects had to exercise fourteen days under supervision and the FGF21 concentrations increased significantly in the cohort (28).

Pharmacological administration of FGF21 shows specific effects in the liver, the pancreas, the adipose tissue and the central nervous system in mice (8). Therapeutic administration of FGF21 increases insulin sensitivity and energy consumption, which leads to body weight reduction and beneficial effects on glucose and lipid metabolism in obese mice (29). In diabetic mice, FGF21 leads to a higher survival rate of beta cells, higher insulin concentrations in the pancreatic cells and an improvement of glucose metabolism (19).

There are also studies available which show a protecting effect of FGF21 concerning acinar cell survival during pancreatitis in mice. FGF21 lowers pancreatic inflammation and fibrosis through autocrine or paracrine signalling (18). Moreover, the administration also results in lower lipid aggregation in the liver of diet-induced obese mice (30).

Kaess et al. found associations between low-density lipoprotein cholesterol (LDL-C) and genetic variations of the FGFR (FGF-receptor) 2 in European people. They also found associations between higher body mass index (BMI) and a genetic variant of KLB (31).

One research group performed a randomized, double-blind and placebo-controlled study in obese subjects with diabetes mellitus type 2, where they wanted to test the metabolic effects of an FGF21 analogue for 4 weeks compared to placebo. The pharmacological administration of this analogue reduced LDL cholesterol and triglycerides significantly, increased HDL (high-density lipoprotein) cholesterol and led to a body weight reduction. Also, an amelioration of fasting insulin levels was detected, which could potentially explain higher insulin sensitivity. Fasting glucose levels were also lower in the treatment groups, but those changes were statistically not significant (32).

All in all, FGF21 has widespread effects on human metabolism including adipose tissue, the pancreas, muscles, the liver and the brain. Moreover, its expression is stimulated by many factors such as high free fatty acid concentrations, starvation, glucose among others. *Figure 1* summarizes some important information concerning human metabolism and FGF21 (33).

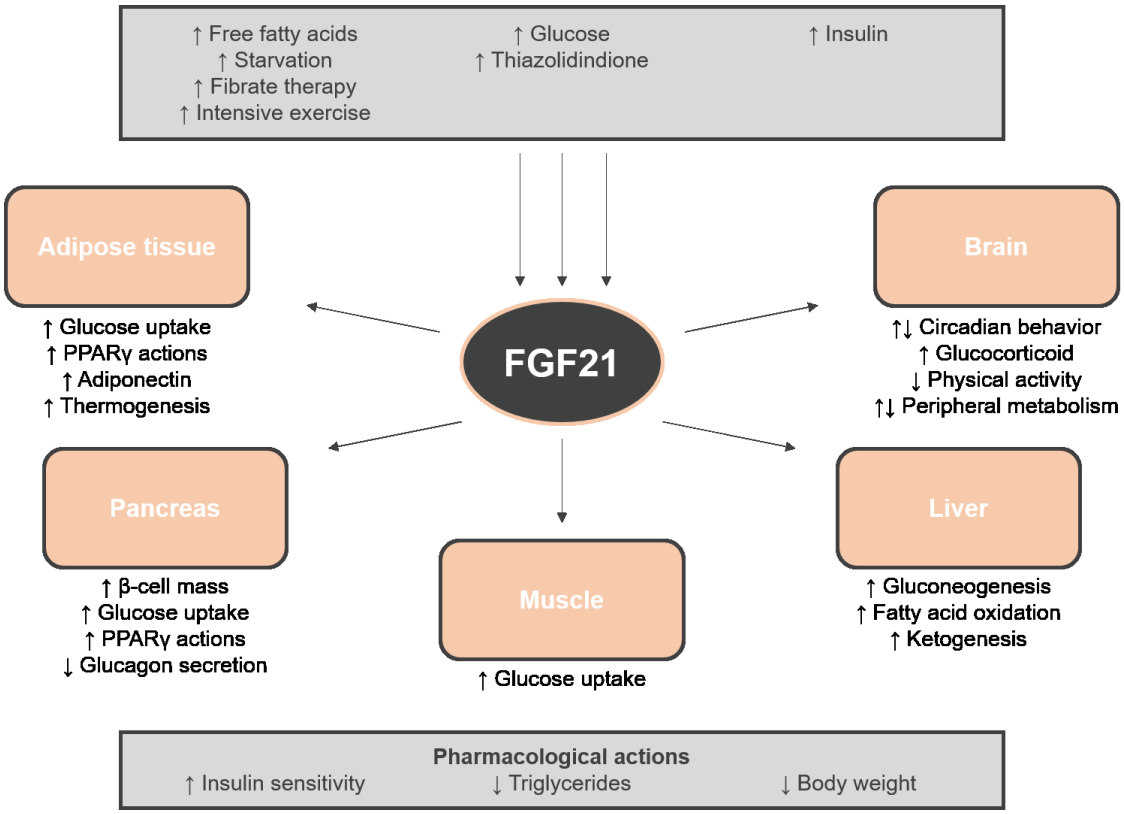


Figure 1: FGF21 and human metabolism

(adapted from Cheung and Deng (33))

Altogether, those results show that a substance that mimics activities of FGF21 could be a potential medical drug for the prevention and therapy of the metabolic syndrome and some of his features, like obesity, hyperglycaemia or dyslipidaemia.

1.2.3 FGF21-resistance in obese people

Although FGF21 has many positive features concerning human metabolism, for example lipolysis, studies found a positive correlation between the body mass index or obesity and levels of FGF21 in humans (34-36). An explanation for this paradox could be FGF21-resistance in obese people. Fisher and his colleagues found out that the expression of β-Klotho in the white adipose tissue is much lower in obese mice (no changes in the liver) compared to lean littermates. Moreover, the FGF-receptors 1, 2 and 3 in the white adipose tissue and the FGF-receptor 1 in the liver are also lower expressed in those mice (37).

Furthermore, obesity leads to a chronic inflammation of the white adipose tissue. This circumstance is mediated, among others, by cytokines like tumor necrosis factor alpha (TNFα) as well as endoplasmatic reticulum stress (38, 39). Diaz-Delfin et al. show that TNFα prevents the expression of β-Klotho in the adipose tissue and that it inhibits FGF21-signalling there (38). Moreover, So et al. found that high glucose levels decrease β-Klotho expression, as well as PPARγ expression, in the pancreatic islet cells. This depression of β-Klotho and PPARγ expression through continuing high glucose levels under diabetic conditions leads later to FGF21-resistance in the islets of Langerhans (40). Those studies about FGF21-resistance were based on another work where Mraz et al. found out, that baseline FGF21 concentrations were elevated in human subjects with diabetes mellitus type 2 (41).

Hale et al. found that this FGF21-resistance in the white adipose tissue is not given when FGF21 is administered pharmacologically at levels that are 1000-fold greater than the normal blood levels in mice. With this FGF21 administration, the expression of β-Klotho and the FGF-receptor 1 in the white adipose tissue was not downregulated, indicating that maybe low β-Klotho-expression leads to resistance (42). As a consequence thereof, higher β-Klotho-expression may prevent FGF21-resistance. Exenatide, a glucagon like peptide-1 receptor agonist that normally helps to control blood glucose levels in patients with type 2 diabetes mellitus, for example, managed to improve some signs of FGF21-resistance in the liver of diabetic patients (43).

1.3 Important previous studies

1.3.1 Fructose ingestion strongly increases levels of FGF21

High ingestion of fructose leads to obesity and related metabolic problems, like glucose intolerance, fatty liver disease or dyslipidemia in humans (44). The carbohydrate response element binding protein (ChREBP) is a transcription factor, which is activated by products of the carbohydrate metabolism and which is really important in the regulation of fructose and glucose metabolism (45, 46). The liver and the intestine show the highest ChREBP expression in mice (47). Von Holstein-Rathlou et al. show in their experiments that sugar consumption in mice activates ChREBP and this leads to FGF21 production in the liver (48).

Dushay et al. report that there is also a significant elevation of serum FGF21 levels in humans after ingestion of 75 grams (g) of fructose, with a peak at 120 minutes. Moreover, they show that there is a difference between the baseline levels of FGF21 in healthy subjects and subjects with metabolic syndrome. Also, the increase in subjects with metabolic syndrome is stronger (49). If ChREBP is also responsible for this increase of FGF21 in humans needs to be investigated.

Soberg et al. tried to find an association between variants in the gene locus of FGF21 and sweet preference in the Danish Inter99 cohort with approximately 6500 participants. The locus FGF21 rs838133 was statistically significant associated with high sweet intake, measured by a detailed food frequency questionnaire. Also, a notable association between the locus and smoking behavior and alcohol consumption was detected. In a second trial in this study they found out that plasma FGF21 levels were significantly increased in fasted subjects, who did not like candy, compared to “sweet-likers” (50).

1.3.2 Glucose ingestion weakly increases levels of FGF21

Oral glucose tolerance tests (oGTT) are a useful tool to identify diabetes mellitus or gestational diabetes. Blood sugar concentrations are quantified after ingestion of 75 g of glucose dissolved in 250 millilitres (ml) of water. When the blood sugar levels reach a specific limit, there might be something wrong with the glucose metabolism (51).

The relationship between oral glucose tolerance tests and serum levels of FGF21 are differentially described in literature. Dushay et al., who had also performed the fructose test mentioned before, for example, in one study didn’t find any significant decrease or increase of FGF21 levels after a 75 g oral glucose load in their 20 healthy, normal weight subjects after 16 hours of fasting. Blood samples were taken for 5 hours (34). In contrast, in another study, they also compared FGF21 levels after ingestion of 75 g of fructose with the ingestion of 75 g of glucose in healthy subjects and subjects with the metabolic syndrome for 4 hours. The increase after the oral glucose load was delayed, in comparison to the oral fructose load, and reached its peak, which was approximately 40% lower, at 240 minutes. Again, the increase in subjects with the metabolic syndrome was stronger (49).

1.3.3 Alcohol preference correlates with levels of FGF21

Alcohol-related disorders are very common in modern society. There are differences between alcohol-use and alcohol-induced disorders (52). Former consists of alcohol dependence and abuse, latter consists of alcohol intoxication, alcohol-induced sexual disorders and many other disorders (52). Chronic heavy drinking is responsible for several severe diseases, as for example liver cirrhosis, pancreatitis, different carcinomas, epilepsy or even diabetes mellitus. Also important to mention are alcohol related accidents, rapes and injuries (53).

Talukdar et al. show that FGF21 treatment reduces alcohol preference in mice. They also show that there is a correlation between levels of dopamine in the Nucleus accumbens (the human reward centre), levels of FGF21 and the desire for alcohol in rodents (9). Schumann et al. show that the transmembrane protein β-Klotho in the brain is responsible for alcohol preference. Mice lacking β-Klotho in the brain have stronger desire for alcohol, compared to wild type littermates. Schumann et al. also show that FGF21, relating to alcohol preference, acts directly on the brain (23). Desai et al. found that acute or binge alcohol consumption increases FGF21 levels in humans and mice with a peak at 6 hours (54). Therefore, it seems that FGF21 could be a potential pharmacological target to control alcohol consumption in humans.

1.3.4 The effect of fat on FGF21 levels is undetermined

One group compared the impact of carbohydrates and fat on plasma FGF21 levels in humans. While carbohydrates increased plasma FGF21 levels significantly compared to a control diet, fats did not (though, a trend was detectable). This is a hint that a high-caloric diet by itself is not the reason for FGF21 expression (55).

Another study showed in mice that the addition of fat as a lipid emulsion to a high-carbohydrate diet even manages to suppress the high increase of FGF21 concentrations through the carbohydrates (56). Chapnik et al. even detected decreased hepatic FGF21 mRNA (messenger ribonucleic acid) levels after a high-fat diet in mice (57).

In contrast, Sun et al. reported different findings in mice. They fed their mice also with a high-fat diet and measured not only FGF21 mRNA expressions, but also expressions of the transmembrane protein β-klotho and the three FGF-receptors 1, 3 and 4. All values were increased after the high-fat diet in the liver and the adipose tissue except FGFR4 mRNA (58). However, FGF21 is not able to bind to this FGF-receptor 4 (16). Plasma, liver and adipose tissue FGF21 protein concentrations were also significantly elevated (58).

1.3.5 Protein restriction elevates FGF21 concentrations in humans

Laeger et al. performed some experiments concerning rodent and human FGF21 concentrations and protein restriction. Already after one day of a low-protein diet, hepatic Fgf21 expression and circulating FGF21 levels were strongly elevated in mice. In humans they also detected a strong increase of plasma FGF21 concentrations after 4 weeks of protein restriction (59). Another group also confirmed this hypothesis. They also found a statistically significant increase of Fgf21 gene expression in hepatic tissue after a low-protein diet in mice. In their human study they found a negative correlation between protein intake (quantified with a food frequency questionnaire) and plasma FGF21 concentrations. Each gram of protein decreased FGF21 concentrations by approximately 3.4 pg/ml (picogram/millilitre) (60).

Lees et al. performed similar studies on mice, but they even tested a more specific protein restriction. They restricted the consumption of the essential amino acid methionine in their rodents and also detected a strong increase of both liver Fgf21 gene expression and serum FGF21 levels. They also found some positive effects on the metabolism of their mice through methionine restriction, like body weight reduction, amelioration in glucose tolerance or lipid metabolism. Those findings indicate a potential relationship between methionine restriction, FGF21 and certain metabolic pathways (61).

Chalvon-Demersay et al. compared a high-carbohydrate low-protein diet with a low-carbohydrate high-protein diet in mice. The high-carbohydrate diet induced hepatic Fgf21 gene expression, whereas the high-protein diet did not. Moreover, they tested the impact of glucose and amino acids on liver cells directly. Only glucose induced FGF21 secretion in the hepatocytes (62).

1.4 Metabolic Syndrome

The metabolic syndrome is no disease by itself, it is a collection of various criteria concerning metabolic disorders (63). If humans consume too high amounts of simple sugars and fat and/or do too little physical activity, in many cases insulin resistance is the result, because of various cell and tissue adaptations. Insulin resistance then leads to an increase of insulin concentrations in the blood and later to an increase of blood glucose levels. One frequent result is the development of diabetes mellitus type 2 (2).

In further consequence, insulin resistance in the adipose tissue leads to an increase of free fatty acid production, lower bioavailability of HDL and higher compactness of LDL through various mechanisms. This disorder of lipid metabolism, as a result of insulin resistance, is a main risk factor for cardiovascular diseases (64). Insulin resistance also facilitates the development of early atherogenesis through dysfunction of the arterial epithelial cells, disturbed dilatation and invasion of inflammatory cells into the epithelium (65). Furthermore, there is a statistically significant association between hyperinsulinemia and the risk for hypertonia (66). The mechanisms behind the insulin dependent elevation of the blood pressure are multifarious (67-72).

Altogether, those metabolic derailments are potential factors for the development of atherogenesis and therefore the reason for the high morbidity and mortality in patients with the metabolic syndrome (2, 63, 73).

The metabolic syndrome consists of various metabolic disorders such as obesity, dyslipidaemia, hypertension or insulin resistance / diabetes mellitus type 2 *(Table 1)*.

Table 1: Classification criteria of the metabolic syndrome [from (2)]

3 of those 5 criteria are needed for the definition of the metabolic syndrome:		
I	waist circumference	>102 cm for men, >88 cm for women
II	triglycerides	≥150 mg/dl *or* therapy with fibrates or niacin
III	HDL cholesterol	<40 mg/dl for men, <50mg/dl for women *or* therapy with fibrates or niacin
IV	blood pressure	≥130/85 mmHg *or* therapy with antihypertensive drugs*
V	plasma glucose	≥100 mg/dl *or* diabetes mellitus *or* therapy with antidiabetic drugs**
* such as ACE (Angiotensin-converting enzyme) inhibitors, AT1 (Angiotensin II type 1) receptor blockers, beta blockers, diuretics, calcium channel blockers, etc. ** such as metformin, sulfonylureas, glinides, thiazolidinediones, insulin, etc.		

1.4.1 Therapy of the metabolic syndrome

The therapy of the metabolic syndrome is very widespread, because it consists of many different metabolic disorders. Probably, the most important “treatment” to reduce the onset of the metabolic syndrome and its high risk for cardiovascular morbidity and mortality and diabetes mellitus is primary prevention: adapted nutrition, body weight reduction and physical activity (2, 74). Two studies showed that a body weight reduction of at least 5% and any type of regular physical activity (such as walking, sports, gardening, household work) can reduce the risk for subjects with impaired glucose tolerance to progress to manifest diabetes mellitus significantly (75, 76).

Pharmacological substances to reduce the risk for cardiovascular diseases in subjects with metabolic syndrome are statins, antidiabetic and antihypertensive drugs, acetylsalicylic acid and many more (2). Statins are used today to reduce pathologic LDL cholesterol levels to individual goal levels depending on the risk profile. If statins do not work adequately, fibrates and niacin are potentially effective (74). Beside Metformin, also acarbose (another anti-diabetic drug) seems to be very successful in treating subjects with impaired glucose tolerance to reduce the risk for cardiovascular events and hypertension successfully (75, 77).

Currently, the only licensed drug for weight reduction in obese patients is orlistat. It blocks the enzyme lipase in the pancreas. This leads to a fat malabsorption and a reduction of fat resorption of 30% in the gastrointestinal tract (2). Orlistat also reduces levels of HbA1c and therefore the onset of diabetes mellitus because it also reduces lipid and glucose concentrations in the blood (78, 79).

1.4.2 Metabolic Syndrome and FGF21

Although lifestyle changes seem to be very effective in the primary prevention of diabetes mellitus and cardiovascular diseases in patients with the metabolic syndrome, it is an unmet clinical need to find potential pharmacological therapies to prevent the desire for simple sugars and therefore the development of metabolic disorders even earlier – keyword: FGF21!

Zhang et al. compared serum FGF21 concentrations in obese subjects with lean subjects. Not only the baseline levels were significantly higher in overweight subjects, but also correlations between serum FGF21 levels and levels of insulin and triglycerides were found. Moreover, an independent association between concentrations of FGF21 and the metabolic syndrome is described.

FGF21-resistance can potentially explain this paradox (35). In obese people FGF21 levels are elevated because of high free fatty acid and glucose concentrations and maybe because of β-Klotho-downregulation (8, 38, 49). FGF21-resistance in obese people is an important circumstance, as described in *1.2.3 (FGF21-resistance in obese people)*. Despite that, pharmacological treatment with FGF21 analogues like LY2405319 seem to play a fundamental and promising role in treatment of the metabolic syndrome, as described in *1.2.2* (*FGF21: systemic and tissue-specific effects)* (32).

1.5 Alcoholic disease

In general, the earlier young people begin to drink, the earlier alcohol associated diseases will occur. The number of hospital stays in Austria for people, who were 14 years or younger, was 39/100.000 residents in 2016 because of alcohol intoxication. Although this number seems to be quite high, there is a constant decline since 2008 when the numbers were as high as 100/100.000 residents (80). The reason for this decline is quite unclear, but it is definitely a positive trend.

According to a survey, the average alcohol per capita consumption in Austria (15 years and older) in 2015 was in total 27 grams of pure alcohol per day *(Table 2)*.

Table 2: Total alcohol per capita consumption in Austria in 2015 [from (81)]

	alcoholics	non-alcoholics	total
men and women			
average grams of pure alcohol per day	200*	16	27
average litres of pure alcohol per year	92.5	7.8	12.5
men			
average grams of pure alcohol per day	226	26	40
average litres of pure alcohol per year	104.6	12.2	18.8
women			
average grams of pure alcohol per day	130	7	13
average litres of pure alcohol per year	60.1	3.4	6.2
**200 g of pure alcohol correlate with approximately 2.5 litres of wine or 5 litres of beer*			

However, in literature there are some hints that alcohol can also prevent coronary heart diseases. The French paradox explains the low rates of cardiovascular events in France inter alia through their moderate consume of alcohol (10 to 30 g pure alcohol per day [from (2)]) – particularly red wine. Alcohol leads to an increase of HDL cholesterol, which potentially explains the beneficial effects of the French paradox (82, 83). Though, there is also evidence that the protective effect of wine drinking could be explained by the higher social states of their consumers (2).

Polsky and Akturk found also beneficial effects of light to moderate alcohol consumption concerning development of diabetes mellitus. In the two Dutch cohorts, for example, moderate drinking (defined as 5 to 29.9 g of pure alcohol per day) was associated with lower incidence of diabetes. In contrast, heavy (in the Finnish cohort defined as >30 g of pure alcohol per day for men and >20 g of pure alcohol per day for women) and binge drinking was associated with an increased risk of diabetes (84).

1.5.1 Definitions

The former term “alcoholism” was replaced by “alcoholic disease” (2). A distinction is drawn between risky alcohol consumption, harmful alcohol abuse and alcohol dependence *(Table 3)*.

Table 3: Classification of alcoholic disease [from (2)]

Important definitions of alcoholic disease
risky alcohol consumption: alcohol consumption, which is associated with harmful effects on the human health, according to epidemiologic studies; this critical value is 40 g of pure alcohol per day for men and 20 g of pure alcohol per day for women; though, harmful effects are also seen in lower doses
alcohol abuse (harmful use): drinking behaviour, which leads to physical and psychosocial health damage
alcohol dependence: 3 of those 8 criteria need to be fulfilled: - strong desire/compulsion - lower control to stop drinking - abstinence phenomenon symptoms - consumption to decrease symptoms - tolerance development - drinking without any occasion - neglect of interests - consumption despite harmful effects

1.5.2 Alcohol overconsumption and health risks

As already mentioned, light to moderate alcohol consumption can have positive effects on human health, particularly in older subjects (85). Excessive alcohol consumption, however, can harm the human body in multiple ways. The most relevant cause of death through alcohol consumption is alcoholic liver disease. Alcohol use disorders are still the most important reason for the onset of liver cirrhosis in European people (86). Alcohol overconsumption often leads to liver cancer (87). Other alcohol related cancers are, among others, breast cancer, diverse cancers of the upper gastrointestinal tract (like mouth, pharynx, larynx, oesophagus and stomach cancer) and pancreatic carcinoma (88-90).

Moreover, also the incidence of the metabolic syndrome and therefore the risk for cardiovascular diseases and diabetes mellitus is elevated in subjects who consume too high amounts of alcohol (91). The incidence of ischemic and haemorrhagic stroke is also higher (92). Excessive alcohol consumption is still the most important cause of chronic pancreatitis (90). Other important results of heavy drinking are the fetal alcohol syndrome for unborn child and various dementias, like vascular and Alzheimer dementia (93, 94).

Not only concerning metabolic syndrome, also in case of alcohol use disorders and alcoholic diseases primary prevention is a fundamental need in medicine – again: keyword FGF21!

1.6 Aim of the study

FGF21 plays an important role in animal and human metabolism. We aimed to measure plasma FGF21 levels after diverse oral challenges. We hypothesized that plasma FGF21 concentrations will significantly change after the oral ingestion of alcohol and diverse macronutrients. To record time-dependent alterations, blood samples were taken at various timepoints up to 4 hours.

With our study we want to highlight and confirm the effects of alcohol, sugars and other macronutrients on the Fibroblast Growth Factor 21 in the human organism.

2 Materials and Methods

2.1 Subject selection

My colleagues from the ZMF (Zentrum für Medizinische Grundlagenforschung) and I altogether included 53 healthy volunteers without diabetes mellitus or other metabolic diseases into our study to assess plasma FGF21 levels after oral ingestion of single or mixed macronutrients as well as alcohol. Moreover, 10 diabetic subjects volunteered to participate in a meal tolerance test (MTT) to measure its effect on plasma FGF21 concentrations in a diabetic organism compared to a healthy state.

Informed consent was obtained before the study. The subjects were not allowed to drink alcohol 48 hours before the experiment started and they had to be in a fasted state. Drinking water was allowed. All inclusion and exclusion criteria are shown in *Table 4.*

Table 4: Inclusion and exclusion criteria of the study

Inclusion criteria
participant is willing and able to give informed consent
participant is older than 18 years
participant is willing to abstain from alcohol 48 hours prior to the study visits
Exclusion criteria
participant suffers from alcohol abuse • Alcohol Use Disorders Identification Test ≥ 8 in men or ≥ 7 in women *or* • CAGE* test ≥ 2 (both men and women)
participant has an elevated liver function test
participant has any disease or medication that does not allow concomitant consumption of alcohol
female participants who are pregnant or breast-feeding
**screening test for problem drinking and potential alcohol problems*

2.2 Experimental procedure

After a detailed explanation of the study procedures, including the intake of alcohol under medical supervision, inclusion and exclusion criteria were checked. Measured normal liver function parameters confirm healthy liver status *(Table 5)*.

After they gave written informed consent all subjects had to answer some questions about demographic data, medical and surgical history and concomitant medication. Date of birth, gender and alcohol consumption were determined. Body weight and height was specified. Blood pressure was measured with auscultatory method (stethoscope and sphygmomanometer) or with an electric sphygmomanometer. Subject characteristics, alcohol drinking habits and liver tests are shown in *Table 5*.

Table 5: Subject characteristics | mean (standard deviation) *or* n (%)

	alcohol group (n=15)	**fruit juice group** (n=8)	**fructose group** (n=10)	**glucose group** (n=10)	**MTT group (healthy)** (n=10)	**MTT group (diabetic)** (n=10)
age (years)	26.1 (4.0)	32.5 (7.2)	25.3 (3.3)	52.5 (7.9)	22.9 (1.9)	61.4 (5.0)
sex						
male	11 (73%)	2 (25%)	7 (70%)	5 (50%)	3 (30%)	2 (20%)
female	4 (27%)	6 (75%)	3 (30%)	5 (50%)	7 (70%)	8 (80%)
weight (kg)	82.3 (15.6)	70.3 (12.2)	71.7 (13.4)	69.3 (6.0)	65.8 (11.0)	105.0 (14.3)
height (cm)	177.2 (8.2)	171.3 (9.5)	175.3 (8.3)	171.0 (5.7)	174.1 (10.3)	172.5 (8.9)
BMI (kg/m²)	26.4 (5.2)	24.0 (3.2)	23.1 (2.7)	23.7 (1.3)	21.6 (2.2)	35.2 (2.6)
Bili (mg/dl)	0.6 (0.3)	0.5 (0.2)	0.7 (0.3)	0.7 (0.5)	0.6 (0.4)	0.6 (0.3)
AP (U/L)	60.9 (19.5)	49.5 (12.1)	59.3 (16.2)	54.0 (9.0)	61.4 (14.7)	72.6 (25.9)
GGT (U/L)	21.1 (11.1)	14.3 (3.8)	24.0 (20.2)	17.8 (8.1)	13.3 (3.3)	47.2 (14.5)
CHE (kU/L)	8.2 (1.6)	7.1 (1.4)	7.5 (1.1)	8.2 (1.9)	6.9 (0.9)	N/A
AST (U/L)	31.9 (17.4)	20.5 (4.0)	30.6 (10.0)	28.6 (7.8)	27.1 (8.1)	28.1 (9.0)
ALT (U/L)	27.0 (14.6)	20.1 (9.2)	32.6 (19.7)	21.6 (6.6)	20.3 (7.2)	31.7 (11.5)
Alcohol consumption (U/week)	1.9 (1.3)	1.8 (1.6)	2.2 (1.5)	N/A	3.9 (2.7)	N/A
Nature of alcohol						
wine	9 (60%)	4 (50%)	5 (50%)	N/A	7 (70%)	N/A
beer	13 (87%)	4 (50%)	9 (90%)	N/A	7 (70%)	N/A
spirits	3 (20%)	2 (25%)	1 (10%)	N/A	5 (50%)	N/A

ALT=alanine transaminase; AP=alkaline phosphatase; AST=aspartate transaminase; Bili=bilirubin; BMI=body mass index; CHE=cholinesterase; cm=centimetre(s); dl=decilitre(s); GGT=gamma glutamyltransferase; kg=kilogram(s); kU=kilounit(s); L=litre(s); m²=square metre(s); mg=milligram(s); MTT=meal tolerance test; n=number; N/A=not available; U=unit(s)

After blood was taken for the baseline plasma FGF21 levels the subjects received diverse oral solutions to drink followed by time-dependent measurements of plasma FGF21 levels.

15 subjects (11 men and 4 women between the ages of 21 and 38) received 2 ml of 40% vodka per kilogram (kg) body weight mixed in a total volume of 300 ml with orange or strawberry juice. 8 more subjects (2 men and 6 women between the ages of 21 and 42) were in the control group and received 2 ml water per kg body weight mixed in a total volume of 300 ml again with orange or strawberry juice without alcohol. The time limit for the consumption of either alcohol or fruit juice was 30 minutes.

10 healthy subjects (7 men and 3 women between the ages of 21 and 32) received 75 g of fructose dissolved in 300 ml of tap water. 10 further subjects (5 men and 5 women between the ages of 35 and 63) performed an oral glucose tolerance test, where they received 75 g of glucose dissolved in 300 ml of tap water. A meal tolerance test was performed with 10 healthy volunteers (3 men and 7 women between the ages of 19 and 27) without metabolic diseases and 10 diabetic subjects (2 men and 8 women between the ages of 50 and 69). They consumed 10 kilocalories (kcal) per kg bodyweight of a mixed caloric drink (Nutricia Fortimel® Compact Protein coffee or vanilla flavor). 100 ml of this mixed caloric drink include 9.4 g fat, 24.4 g carbohydrates, 14.4 g proteins and 240 kcal. The time limit for the consumption of those drinks was 5 minutes.

Blood samples were taken after 60, 120, 180 and 240 minutes in the alcohol and fructose group and the healthy MTT cohort. As standard operating procedures in the oGTT and diabetic MTT cohort blood samples were only taken after 30, 60 and 120 minutes. Blood was taken via an intravenous catheter. Some blood was sent to the local laboratory to measure diverse standard blood parameters *(Table 5)*. A pregnancy test in women of childbearing age was also done. After centrifugation lithium heparin plasma samples were stored at -80°C.

Plasma FGF21 levels were measured by a human FGF21-ELISA-kit. Details are shown in *2.3 (Human FGF21 ELISA)*.

2.3 Human FGF21 ELISA

In all experiments, human plasma FGF21 levels were quantified with the RD191108200R HUMAN FGF-21 ELISA (Enzyme-linked Immunosorbent Assay) kit manufactured by BioVendor – Laboratorní medicína a.s. (Brno, Czech Republic). It is a sandwich enzyme immunoassay for the quantitative measurement of human FGF21. Components of the kit are provided ready to use. FGF21 is measured in plasma by ELISA according to the manufacturer's instructions.

Plasma samples were diluted 1:2 with dilution buffer just prior to the assay. Then the samples were mixed with Vortex. 100 microlitres (µl) of standards, reconstituted quality controls and diluted samples were pipetted into FGF21 antibody coated microtiter wells. The plate was incubated at room temperature for one hour on an orbital microplate shaker at 300 rounds per minute (rpm). Then the wells were washed three times with 0.35 ml of wash solution per well and the inverted plate was tapped strongly against a paper towel. Next, 100 µl of biotin labelled FGF21 antibody were pipetted into each well. After incubating the plate for another hour at room temperature on the orbital microplate shaker at 300 rpm, the next washing step as mentioned above was performed. 100 µl of streptavidin horseradish peroxidase (HRP) conjugate were pipetted into each well, followed by a 30 minutes incubation period on the orbital microplate shaker at room temperature and 300 rpm and the next washing round. After 100 µl of substrate solution were added, exposition to direct sunlight was avoided and the plate was incubated at room temperature for 15 minutes without shaking. The color reaction was stopped by adding 100 µl of a stop solution. Within 5 minutes, the absorbance of each well was measured by a microplate reader set to 450 nanometers. The concentration of human FGF21 was calculated from the standard curve and multiplied by the dilution factor, to reach the right values (picogram/millilitre = pg/ml).

Sensitivity or limit of detection in this kit is 7 pg/ml. Limit of assay are plasma FGF21 levels of 1920 pg/ml and more. The antibodies in this ELISA are specific for human FGF21.

2.4 Ethical consideration

The Ethics Committee of the Medical University of Graz approved both of the study protocols (26-464 ex 13/14 and 28-255 ex 15/16). The study was performed according to good clinical practice principles. Some ethical issues needed to be considered. One serious question was how to perform a clinical study with drinks with high alcoholic content in healthy subjects for a duration of more than four hours. Appropriate rooms and continuous supervision needed to be given. Moreover, throughout all experiments the numerous time-dependent blood draws after diverse oral challenges were complex and needed to be prearranged exactly. Also, the potential side effects of the high concentrated drinks, like 75 grams of fructose or glucose dissolved in water, on the gastrointestinal tract were quite unknown and this needed to be mentioned in the informed consent of the study. In summary, many ethical issues appeared during study planning but were constantly handled adequately.

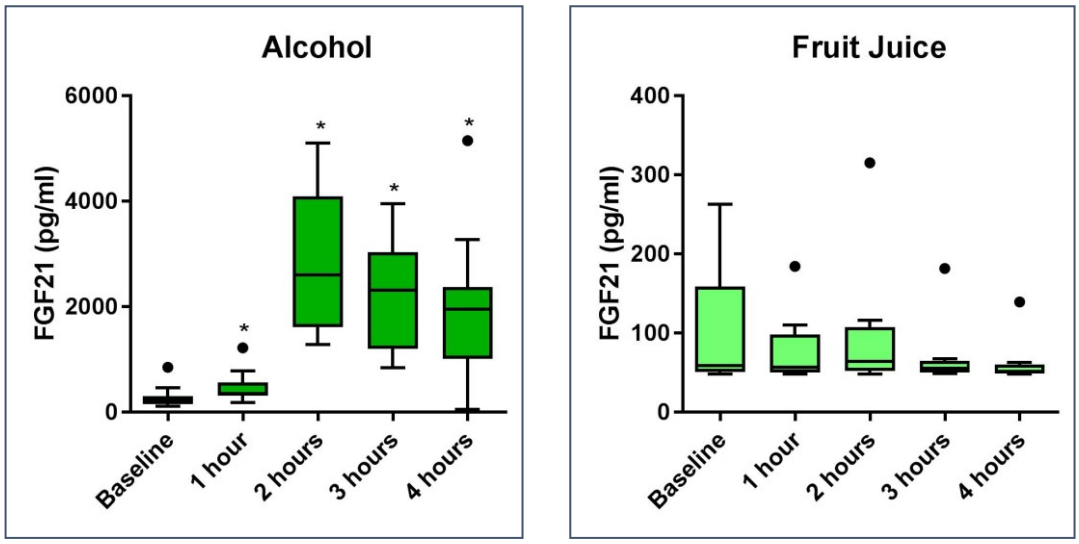
2.5 Statistical analysis

IBM SPSS Statistics 23 and 25 and GraphPad PRISM 6 and 7 were used for statistical analysis. All graphs were created with GraphPad PRISM 6 and 7. Microsoft Excel 2016 was used to record the data of the subjects. For statistical analysis of the FGF21 changes after oral challenges, Friedman test was performed. Dunnett corrected p-values were significant <0.05. To compare baseline levels between the different cohorts Kruskal-Wallis and Dunn's multiple comparisons test were performed. The same statistical analyses were done to compare the different elevations and declines two hours after the oral challenges compared to baseline. Adjusted p-values were significant <0.05. To highlight the higher baseline FGF21 levels of the diabetic cohort in comparison to the healthy control group and to demonstrate the robustness of the difference, a Mann-Whitney-U test was performed.

3 Results

3.1 Alcohol ingestion acutely increases plasma FGF21 levels

Figure 2 shows the plasma FGF21 changes after alcohol ingestion in the intervention group *(left)* and the plasma FGF21 changes after fruit juice ingestion in the control group *(right)*. Alcohol induced a significant increase of plasma FGF21 levels after 1, 2, 3 and 4 hours (h) compared to baseline. The peak was reached at 120 minutes, with a 10.3-fold increase (p=0.001). In contrast, in the fruit juice group there were no chances of plasma FGF21 levels detectable.



*p-value after Dunnett correction <0.05
Note: each graph has different scales on its vertical Y-axis

Figure 2: Plasma FGF21 changes in alcohol (n=15) and control group (n=8)

Below, the measured data from the human FGF21-ELISA-kit is shown *(Table 6* and *Table 7).*

Table 6: Plasma FGF21 changes (pg/ml) in alcohol group (n=15)

	Baseline	1h	2h	3h	4h
Median	221.95	356.57	2600.27	2317.86	1958.95
Minimum	112.36	181.99	1282.16	847.04	59.00
Maximum	852.71	1223.57	5100.70	3955.92	5154.33
Range	740.35	1041.58	3818.53	3108.88	5095.33
Mean	275.89	481.72	2845.61	2178.78	1913.74
Standard deviation	187.54	261.25	1255.08	979.43	1174.10

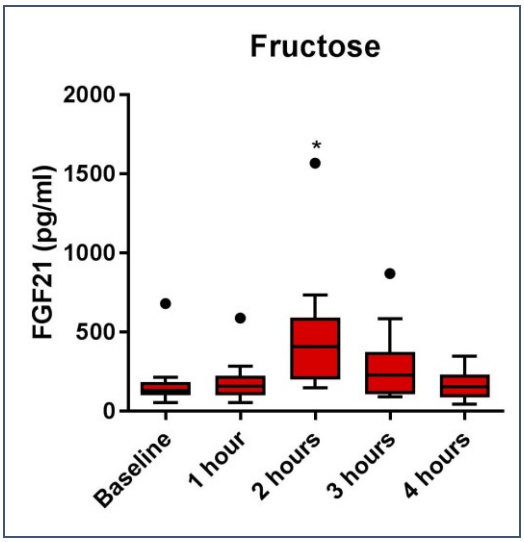
Table 7: Plasma FGF21 changes (pg/ml) in fruit juice group (n=8)

	Baseline	1h	2h	3h	4h
Median	59.02	57.13	64.21	55.70	51.22
Minimum	48.02	48.21	48.11	48.95	48.18
Maximum	263.16	184.39	315.44	181.87	139.54
Range	215.14	136.19	267.33	132.92	91.37
Mean	99.96	77.88	99.43	71.12	63.28
Standard deviation	74.79	261.25	84.32	42.23	29.14

3.2 Fructose ingestion acutely increases plasma FGF21 levels

Figure 3 shows the changes of plasma FGF21 levels in 10 healthy subjects after fructose ingestion. A significant 2.7-fold increase of plasma FGF21 levels after fructose ingestion at 2 hours compared to baseline was seen (p=0.005). After the peak at 2 hours the levels at 3 and 4 hours did not significantly differ from baseline levels anymore.

The comparison between the alcohol and fructose challenge shows that the increase of FGF21 at two hours after alcohol ingestion is much stronger compared to fructose. However, due to lack of power this did not reach statistical significance. Details are shown in *Figure 6 (chapter 3.5).*



*p-value after Dunnett correction <0.05

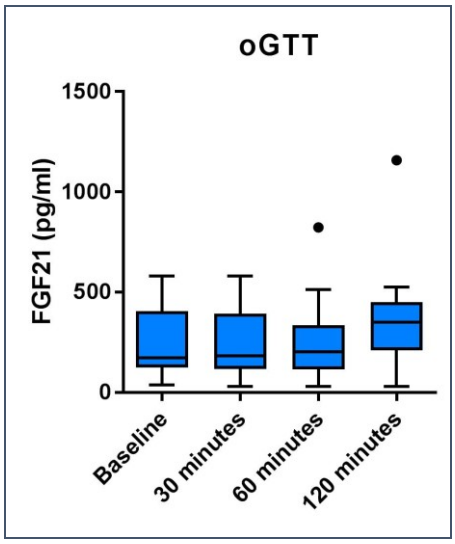
Figure 3: Plasma FGF21 changes in fructose group (n=10)

Table 8: Plasma FGF21 changes (pg/ml) in fructose group (n=10)

	Baseline	1h	2h	3h	4h
Median	132.35	159.90	406.80	229.00	153.65
Minimum	54.20	55.40	149.10	93.30	46.40
Maximum	680.60	588.60	1569.60	870.20	347.70
Range	626.40	533.20	1420.50	776.90	301.30
Mean	185.74	193.68	492.74	293.43	167.38
Standard deviation	171.14	145.97	398.98	237.38	97.97

3.3 Glucose ingestion potentially increases plasma FGF21 levels

There were no significant changes in plasma FGF21 levels found after an oral glucose tolerance test within 120 minutes in 10 healthy subjects. Neither an increase nor a decrease of plasma FGF21 levels were detectable after oral ingestion of 75 g of glucose. Potentially, a light increase of plasma FGF21 levels at 120 minutes was ascertainable *(Figure 4)*.



*p-value after Dunnett correction <0.05

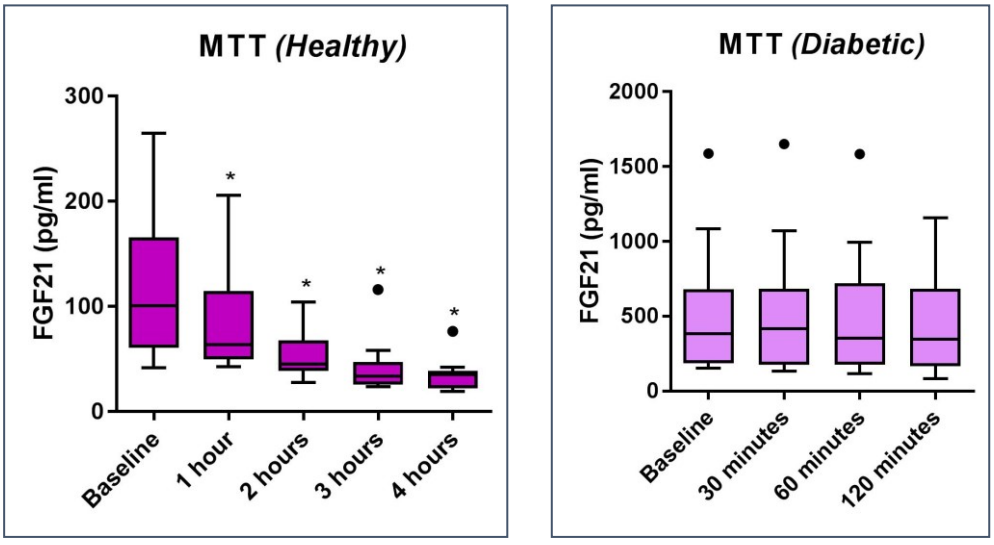
Figure 4: Plasma FGF21 changes in oral glucose tolerance test group (n=10)

Table 9: Plasma FGF21 changes (pg/ml) in oGGT group (n=10)

	Baseline	30min	60min	120min
Median	173.07	181.91	201.52	348.78
Minimum	38.03	29.00	29.00	30.96
Maximum	581.10	578.94	823.55	1158.11
Range	543.07	549.94	794.55	1127.15
Mean	260.30	252.46	266.42	388.98
Standard deviation	180.45	185.59	225.74	292.30

3.4 Meal tolerance test decreases plasma FGF21 levels

After consumption of a mixed caloric drink in the healthy group (n=10) a significant decrease of plasma FGF21 levels every hour within 4 hours compared to baseline was found *(Figure 5; left side)*. After 1, 2, 3 and 4 hours, a 1.4-, 2.2-, 2.7- and 3.4-fold decrease of plasma FGF21 compared to baseline levels was measured, respectively. In contrast, in the diabetic group (n=10) there were no significant changes or trends after the meal tolerance test (MTT) detected *(Figure 5*; *right side)*.



*p-value after Dunnett correction <0.05
Note: each graph has different scales on its vertical Y-axis

Figure 5: Plasma FGF21 changes in healthy (n=10) and diabetic MTT group (n=10)

Table 10: Plasma FGF21 changes (pg/ml) in healthy MTT group (n=10)

	Baseline	1h	2h	3h	4h
Median	100.61	63.71	45.05	33.67	35.24
Minimum	41.75	42.65	27.59	23.84	19.26
Maximum	264.98	205.81	104.15	116.24	76.44
Range	223.23	163.16	76.56	92.40	57.17
Mean	117.48	86.54	53.67	43.07	34.84
Standard deviation	73.54	53.55	23.08	26.50	15.89

Table 11: Plasma FGF21 changes (pg/ml) in diabetic MTT group (n=10)

	Baseline	30min	60min	120min
Median	381.88	415.82	353.63	345.36
Minimum	151.90	132.33	116.28	83.60
Maximum	1587.03	1651.56	1584.90	1159.53
Range	1435.13	1519.23	1468.63	1075.92
Mean	528.83	528.36	516.41	432.87
Standard deviation	441.78	459.74	439.98	328.08

3.5 Both alcohol and fructose strongly stimulate FGF21 production

As has been shown above, alcohol and fructose ingestion lead to a statistically significant release of FGF21 into human circulation at two hours compared to baseline (see also *3.1* and *3.2*). To determine which oral challenge has the highest impact, statistical analyses were done to determine the strongest FGF21 inducer in the study. We compared Δ2-hour-levels (Δ=delta) of each challenge (except MTT diabetic) with each other to calculate significant differences between the increase of FGF21 levels at two hours compared to baseline *(Figure 6)*. Δ2-hour means that we calculated the difference of FGF21 levels between 2 hours and baseline. The results were compared among each other using Kruskal-Wallis one-way analysis of variance by ranks and Dunn's multiple comparisons test.

Alcohol is the strongest inducer of FGF21 production as seen in *Figure 6*. Alcohol ingestion leads to a significant higher increase of plasma FGF21 concentrations after 2 hours compared to fruit juice *(adj. [adjusted] p-value ≤ 0.0001)*, MTT in the healthy group *(adj. p-value ≤ 0.0001)* and glucose *(adj. p-value = 0.0023)*. Moreover, fructose ingestion also leads to a statistically significant increase of plasma FGF21 concentration compared to MTT in the healthy cohort *(adj. p-value = 0.0063)*.

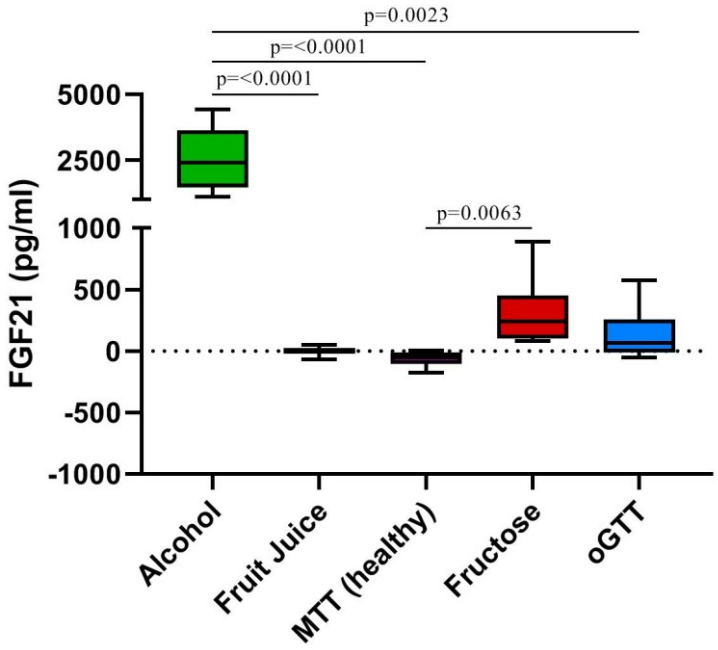


Figure 6: Δ2-hour-levels of each challenge (except MTT diabetic)

Moreover, all progressive curves (except MTT diabetic) were matched into one figure *(Figure 7)*. The figure shows again that alcohol *(orange line)* is the strongest inducer of FGF21 production in our study followed by fructose *(green line)* and glucose *(blue line)*.

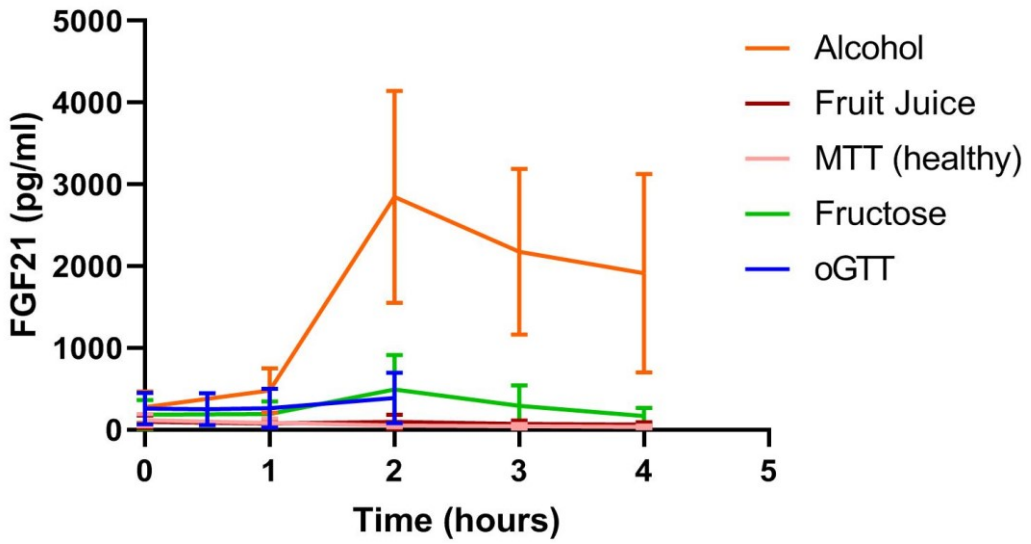


Figure 7: Progressive curves of each challenge (except MTT diabetic)

Apart from alcohol, also fructose and glucose lead to an increase of FGF21 levels two hours after ingestion compared to baseline *(Figure 8)*, although the increase after glucose ingestion is not significant (see also *3.3.*).

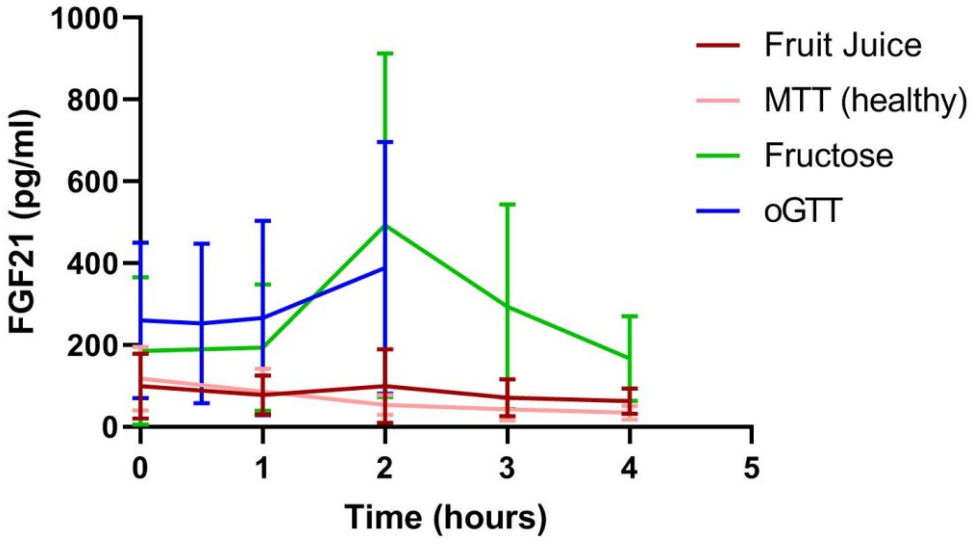


Figure 8: Progressive curves to distinguish fructose, glucose, fruit juice and MTT (healthy)

3.6 Baseline comparisons

Within the MTT challenge 10 diabetic subjects were included. To show that the baseline FGF21 levels in the five healthy groups are the same and, in addition, to find out if they differ in the diabetic subjects from those in the healthy subjects baseline levels of each cohort with each other were compared *(Figure 9).*

In fact, there is a statistically significant difference, which becomes evident using Kruskal-Wallis one-way analysis of variance by ranks and Dunn's multiple comparisons test between baseline FGF21 levels of the diabetic subjects, and the healthy fruit juice cohort *(adj. p-value after Dunn's multiple comparisons test = 0.0025),* and the healthy MTT cohort *(adj. p-value = 0.0063)*.

Moreover, there is also a statistically significant difference found between baseline levels in the alcohol and fruit juice group *(adj. p-value = 0.0425)*, but a batch effect of the ELISA kit could be the reason for this; that means that measurements were potentially affected by laboratory conditions, reagents or personnel differences.

It is important to mention, that throughout the whole experiment the same plates were used constantly. Therefore, the present results can be declared as valid.

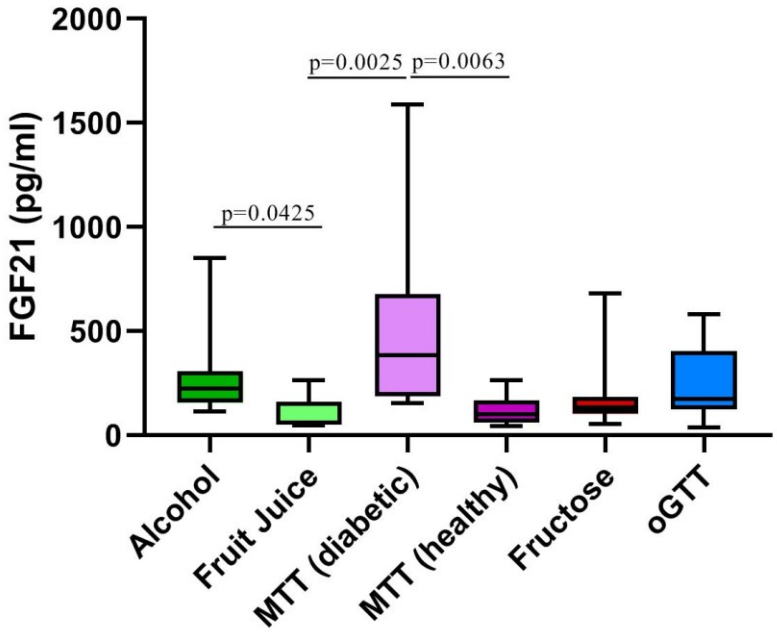


Figure 9: Baseline comparisons of each group

To highlight the robustness and clarity of the higher baseline levels of the diabetic subjects in the two MTT groups (4.5-fold), a Mann-Whitney-U test was performed. P-values were significant ≤ 0.001. *Figure 10* shows this analysis graphically.

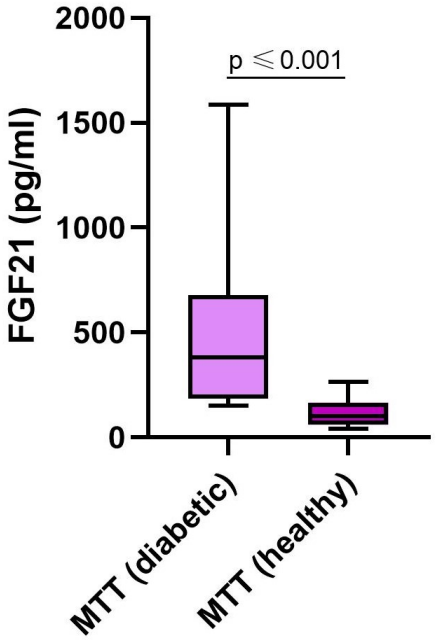


Figure 10: Baseline comparison between MTT diabetic and MTT healthy

3.7 Alcohol drinking habits

Within the questionnaire, which was filled out before the study, the subjects were also asked about their drinking habits to exclude subjects with drinking problems. In the 4 healthy, young groups (alcohol, fruit juice, fructose, MTT healthy) the mean alcohol consumption was 2.4 ± 2.0 units per week. Possible nature of alcohol to choose were beer, wine and spirits. Altogether the most popular drink was beer (76.7%), followed by wine (58.1%) and spirits (25.6%). 9.3% of the subjects in this list do not drink any alcohol at all.

4 Discussion

FGF21 is a well-known hormone, which is affected by plenty of different substances and circumstances, for example alcohol, physical exercise, starvation or insulin, just to name a few (see also *1.2.2* and *1.3.3*).

In this thesis, the aim was to investigate the effect of alcohol, sugars, fats and proteins on human plasma FGF21 levels.

To begin with, experiments with alcohol were done in 15 human subjects. To verify that alcohol by itself is the trigger for FGF21 elevation, the exact same experiment was done in a control group with only fruit juice. In literature, there are several hints that alcohol has the potential to increase levels of FGF21 not only in mice, but also in humans (34, 54). We can confirm those results. In fact, after two hours there was a 10.3-fold increase of plasma FGF21 levels measured in the human FGF21-ELISA-kit. In contrast, there were no changes measured in the control group. This confirms that alcohol alone is responsible for FGF21 elevation.

To some extent, the present results support the findings from Desai et al., who also found an increase of plasma FGF21 levels after alcohol ingestion in their subjects. However, while we saw a significant acute increase of FGF21 levels with a peak at 2 hours, Desai et al. report an increase of FGF21 with a peak at 6 hours (54). Comparison of the amount of alcohol consumed in the different studies show that in our study less alcohol was administered. Our subjects had to consume 2 ml of 40% vodka per kg bodyweight, whereas Desai's subjects had to consume 0.9 g of pure ethanol per kg bodyweight *(75 kg person: 48.0 g versus 67.5 g of pure alcohol, respectively).* To which extent the amount of alcohol plays a role concerning FGF21 production needs to be investigated in future experiments. At the moment, in literature there are no hints detectable to explain that discrepancy.

Next, the role of sugars was tested on human FGF21 levels. Dushay et al. reported a delayed and weaker increase of serum FGF21 levels in healthy subjects after an oral glucose load, compared to a fructose load (49). We can confirm those results. While we saw a statistically significant 2.7-fold increase of plasma FGF21 levels at two hours compared to baseline in our subjects after fructose ingestion, the elevation after the oGTT at 2 hours can only be hypothesized based on a statistical trend.

Unfortunately, our measurements end after 120 minutes at the oGTT. Dushay et al. discovered the peak at 4 hours after glucose ingestion (49). Thereby, we can say that further studies with longer time recordings are needed to address a potential statistically significant elevation.

Important to mention is the mean age of the glucose cohort (52.5 ± 7.9 years), which differs strong from the other healthy cohorts. Despite of that, sex, weight, BMI and liver enzymes are similar compared to the other groups (see also *Table 5*). Because of that, we cannot reduce the delayed and weaker increase of plasma FGF21 levels after glucose ingestion to the weight or BMI. Villarroya et al. found out, that baseline FGF21 levels of patients older than 70 years are increased compared to healthy controls (younger than 40 years; (95)). However, we cannot confirm that there is a difference in baseline levels in our older subjects (see also *3.6*), but age could be also a reason for the delayed and weaker increase of plasma FGF21 levels after the oGTT in our experiment, compared to the fructose load. Future research is needed to clarify this hypothesis.

We also aimed to find the strongest inducer of FGF21 production in our four experimental setups. Therefore, we compared the different elevations of plasma FGF21 levels at two hours compared to baseline. Alcohol reached by far the highest values of plasma FGF21 levels after 2 hours (10.3-fold), but also fructose was a strong inducer (2.7-fold; see also *3.5*).

Fats and/or proteins decrease FGF21 levels in humans. Our results in the MTT indicate that a mixed meal including proteins, fats and sugars lead to a decrease of plasma FGF21 levels, because carbohydrates alone would lead to an increase (see also *3.2* and *3.3*) as seen in the presented work. We found a statistically significant continuous reduction of plasma FGF21 levels at every hour within four hours after ingestion of a mixed caloric drink *(100 ml include 9.4 g fat, 24.4 g carbohydrates, 14.4 g proteins and 240 kcal)* compared to baseline. At 4 hours a 3.4-fold decrease was reached (see also *3.4*). In literature increase of FGF21 levels after protein restriction are often described (59-61). Thereby, we can hypothesize that protein ingestion leads inversely to a decrease of FGF21.

Though, also decrease of FGF21 levels after fat consumption in mice are described in literature (57). Another study in mice reported that the addition of fat as a lipid emulsion to a high-carbohydrate diet even suppresses the high increase of FGF21 concentrations by carbohydrates (56). Those findings potentially accord with ours. However, the question, which nutrient may have a stronger impact on FGF21-signalling, fats or proteins, still remains unanswered.

Another important question that needs to be discussed, is, why the plasma FGF21 levels of the diabetic subjects did not show any relevant changes after the meal tolerance test, compared to the healthy subjects. If we keep in mind, that obese people show insulin resistance, we can try to project this fact on our diabetic subjects (see also *1.2.3*). Comparisons of the weight of our diverse groups show that our diabetic subjects obviously show the highest values, as expected, with a mean BMI of 35.2 kg/m² (Obesity grade II; (2)). So, the static values after meal tolerance test in the diabetic group could be explained by FGF21-resistiance, as consequence from obesity (see also *Table 5: Subject characteristics*). If diabetes itself also causes FGF21-resistance, remains elusive. Talking about FGF21-resistance it is important to highlight that research recently began to find some pharmacologic ways to improve those non-physiological conditions in obese people. But in fact, we need more pharmacological therapies, like Exenatide, to enhance FGF21-sensitivity in the whole human body and in further consequence to prevent FGF21-resistance (see also *1.2.3*).

Moreover, we found out that baseline FGF21 levels of the diabetic cohort show significantly higher values compared to the healthy cohorts (see also *Figure 9* and *Figure 10*). This fact supports the thesis, that diabetic subjects have higher baseline values of FGF21 because of FGF21-resistance. Hence, our subjects do not only suffer from diabetes mellitus, but also from obesity (grade II), which is proven to be a main factor for FGF21-resistance (see also *1.2.3*). Beside those facts, Villarroya et al. found out, that also age plays a fundamental role in higher baseline levels of FGF21 (95). However, maybe more than one aspect is responsible for the higher baseline expression of the hepatokine FGF21 in our diabetic cohort including diabetes, obesity and/or age.

Alcohol and FGF21 strongly interact in many ways. With alcohol habit questionnaires we excluded subjects with alcohol drinking problems (see also *Table 5*). It would be of great interest, if chronic alcohol consumption also leads to higher baseline FGF21 levels or not. With our data, we could not check this hypothesis, but one colleague from the Medical University of Graz, Dr. med. Philipp Grande, did. In his diploma thesis he found out that healthy subjects, who drink at least or more than one unit of alcohol per week, show higher baseline plasma FGF21 levels compared to those, who do not consume any alcohol at all. Unfortunately, his findings did not reach statistical significance (96). Nevertheless, we cannot explain the higher baseline FGF21 levels in the diabetic group with those findings, because we did not determine their alcohol drinking habits.

Looking at the subject characteristics on *Table 5* in general, values like age, sex, weight, height and BMI are, on the whole, very similar between the healthy groups, as expected *(note: as mentioned before, subjects in the oGTT group are older)*. In contrast, the 10 subjects in the diabetic MTT group are older, heavier and have a higher BMI. Liver enzymes are at normal range in every single group, as expected, because healthy liver status was a requirement for study participation (see also *Table 5* in *2.2*).

Although our experiments revealed interesting and distinct results, some evident limitations need to be discussed.

First, it is important to mention that the number of patients in all six experiments is very low (8 to 15 subjects). That instance must be taken into accounts when interpreting the results.

Second, our MTT experiment leaves some questions open. We used a ready-to-drink Fortimel® compact, without any previous well-considered specific mixture of ingredients *(like exactly 75 g of proteins and 75 g of fat)*. Although we know the exact amounts of fats, proteins and carbohydrates from the ingredient directory of the product, we cannot say for sure, which nutrients leaded to those plasma FGF21 decreases. Consequently, more experiments with separated fat and protein challenges are needed to uncover the real metabolic driver for FGF21 level reduction.

However, our results show that some macronutrients and alcohol have remarkable effects on human plasma FGF21 levels. We did not only confirm that sugars have the potential to elevate circulating FGF21, we also found some reducers (fats and/or proteins).

The third limitation to discuss is the short duration of the MTT within the diabetic cohort as well as the oGTT. Per standard operating procedures, those trials were only done for 120 minutes. Particularly longer recordings at the oGTT would have been very interesting, because in literature glucose ingestion leaded to a delayed increase of FGF21 levels compared to fructose ingestion: peaks reached at 240 minutes versus 120 minutes, respectively (49). Our glucose experiment only showed a small trend of FGF21 elevation after two hours compared to baseline – but those results did not reach statistical significance. Longer time recordings are required to detect potential significant results. Moreover, we cannot make a safe statement about the development of FGF21 between two and four hours in our diabetic subjects after the MTT challenge, because we did not take blood samples at those timepoints. Even though it is very unlikely, that those values would change between 120 and 240 minutes after the MTT, it cannot be excluded.

4.1 Conclusion

FGF21 has created immense interest in the last few years. Not only is it related to obesity, metabolic syndrome, diabetes and other diseases, it is affected by many different nutrients in an acute manner. Whether alcohol, sugars, fats and proteins lead to a prompt change of plasma FGF21 production in the liver cells directly, without any intermediate step, remains elusive. However, the question if FGF21 could be a potent pharmacological target to prevent alcohol dependence and diverse features of the metabolic syndrome, might be answered soon.

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