Diploma Thesis

Dietary Effects on Liver and Brown Adipose Tissue Metabolism in Mice

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Graz, April 4, 2022

Statutory Declaration

I hereby declare that I have authored this diploma thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Graz, April 4, 2022 *Dino Hasić eh*

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List of Abbreviations

ACAT	sterol O-acyltransferase
ACLY	ATP citrate lyase
ACSS	acetyl-CoA synthetase
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BAT	brown adipose tissue
BCAA	branched-chain amino acids
BLAST	Basic Local Alignment Search Tool
BLAT	BLAST-like Alignment Tool
CA	cholic acid
CACT	carnitine-acylcarnitine translocase
CDCA	chenodeoxycholic acid
ChREBP	carbohydrate-response element-binding protein
CoA	coenzyme A
CPT	carnitine-acyl transferase
CYP7A1	cholesterol 7 alpha-hydroxylase
CYP27A1	sterol 27-hydroxylase
DAG	diacylglycerol
DGAT	diacylglycerol acyltransferase
DNA/cDNA	deoxyribonucleic acid/complementary deoxyribonucleic acid
DNL	de novo lipogenesis
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
e.g.	exempli gratia
ETC	electron transport chain
F6P	fructose-6-phosphate
FADH2	flavin adenine dinucleotide
FA	fatty acid
FASN (FAS)	fatty acid synthase
FGF	fibroblast growth factor
FXR	farnesoid X receptor

G3P	glyceraldehyde-3-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
GK	glucokinase
GLUT	glucose transporter
GPAT	glycerol-3-phosphate acyltransferase
HFD	high fat diet
HK2 (Hex2)	hexokinase 2
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
Hmgcr (Hmgcoar)	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
Hprt	hypoxanthine phosphoribosyltransferase
IGFBP	insulin-like growth factor binding protein
IGF	insulin-like growth factor
JNK	c-Jun N-terminal kinase
KD	ketogenic diet
LCAT	lecithin-cholesterol acyltransferase
mRNA	messenger ribonucleic acid
mTORC	mammalian target of rapamycin complex
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
PAP	phosphatidic acid phosphatase
PCK	phosphoenolpyruvate carboxykinase
PDH	pyruvate dehydrogenase
PFD	protein free diet
PFKB	phosphofructokinase, B-type
PFKM	phosphofructokinase, muscle
PFK	phosphofructokinase
PKC	protein kinase C
PPAR	peroxisome proliferator-activated receptor
pre-Cyp7a1	Cyp7a1 precursor messenger ribonucleic acid
pre-mRNA	precursor messenger ribonucleic acid
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
SCD	stearoyl-CoA desaturase

SGLT	sodium-glucose linked transporter
SHP	small heterodimer partner
SREBP	sterol regulatory element-binding protein
TAG	triacylglycerol/triglyceride
TCA	tricarboxylic acid
tRNA	transfer ribonucleic acid
VLDL	very-low-density lipoprotein
WAT	white adipose tissue
WD	western diet

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Zusammenfassung

Die Ernährung stellt ein brennendes Thema in der Medizin dar, weshalb deren Einflüsse auf den Organismus und die Genetik heute von vielen Forschern genauer untersucht werden. Die ketogene Diät ist bereits seit den 1920er Jahren in der Therapie von Epilepsie bekannt, seitdem verschiedene Effekte dieser Diät gefunden worden sind. Rezente Studien zeigten, dass mit besonderen Diäten, die Genexpression auf verschiedenen Ebenen beeinflusst werden kann. Weiters ist bekannt, dass ein Gen durch die post-transkriptionelle Prozessierung (z.B. alternatives Spleißen, alternative Promotor Nutzung) der unreifen mRNA für mehrere Gen-Isoformen kodieren kann. Diese Isoformen können auf Proteinebene einzigartige Eigenschaften haben und den Stoffwechsel wesentlich beeinflussen. In dieser Arbeit wurde der Einfluss der ketogenen, aber auch proteinfreien, angereicherten-Fett-Diät und einer „western“ Diät (hoher Cholesterinanteil) auf die Expression der mRNA verschiedener Gene in der Leber und im braunen Fettgewebe der Maus untersucht. Außerdem wurden für Gene, bei denen Gen-Isoformen bekannt sind, diese Isoformen getrennt voneinander untersucht, um neue diätetische Einflüsse auf die Prozessierung der mRNA und somit auch auf den Stoffwechsel finden zu können. Alle Messungen erfolgten quantitativ, mittels qPCR. Diese Arbeit zielt schließlich darauf ab, neue Einflüsse verschiedener Diäten auf den Metabolismus der Leber und des braunen Fettgewebes auf genetischer Ebene zu finden, um diese dann in der klinischen Forschung verwenden zu können. Da es sich um ein sehr breites und komplexes Forschungsgebiet handelt, sind viele Fragen offengeblieben.

Abstract

Nutrition is an important topic in medicine, which has caught the attention of scientists and lead to many studies where nutritional effects on the organism and its genetic properties have been investigated. The ketogenic diet is known since the 1920s in the treatment of epilepsy, but other effects have also been found. Recent studies show that specific diets can influence gene expression at different levels. Furthermore, it is known that a single gene can encode for multiple isoforms, through the post-transcriptional processing of immature mRNA. This process involves alternative splicing or alternative promoter usage resulting in different transcript variants. These transcript variants can have special properties at the protein level and influence the metabolism in different ways. Because these effects are various and their interactions complex, many questions will still remain unanswered. In this diploma thesis, we investigated the effects of the ketogenic, but also a protein free, high-fat-diet and a cholesterol-rich "western diet" on the mRNA levels of various genes involved in the metabolism of the liver and brown adipose tissue. Because there are known isoforms for some of the investigated genes, we designed custom primers with the intention of determining the mRNA levels of these isoforms separately, for more thorough investigation. The data were gathered using quantitative PCR. Thus, the goal of this research was to find novel effects of these diets on the metabolism in the liver and brown adipose tissue at the genetic level, which could prove relevant for upcoming clinical studies.

1 Introduction

1.1 Metabolism and Nutrition

The metabolism of every organism is controlled by various cellular factors in order to provide energy for survival and activity throughout the life cycle of the organism. The nutrients taken into the body needed for the cellular metabolism are sensed and ingested by the cells of the gastrointestinal tract for further salvage, so they can be used for producing energy that the organism needs. The conversion of carbohydrates, lipids, and amino acids into different molecules, accompanied by energy transfer is called “the metabolic flux” and it provides the energy needed for cellular and, on a bigger scale, the organism’s survival [1]. Furthermore, not every organism has the same ability to adapt to a change in dietary intake, which is called “metabolic flexibility” and could be an important factor in disease development [2]. Because diets differ greatly in their composition, they are a remarkable way to change the cellular behavior and their preferred metabolic pathways. It is also known that cellular gene expression can be modified by changing the dietary intake of the organism.

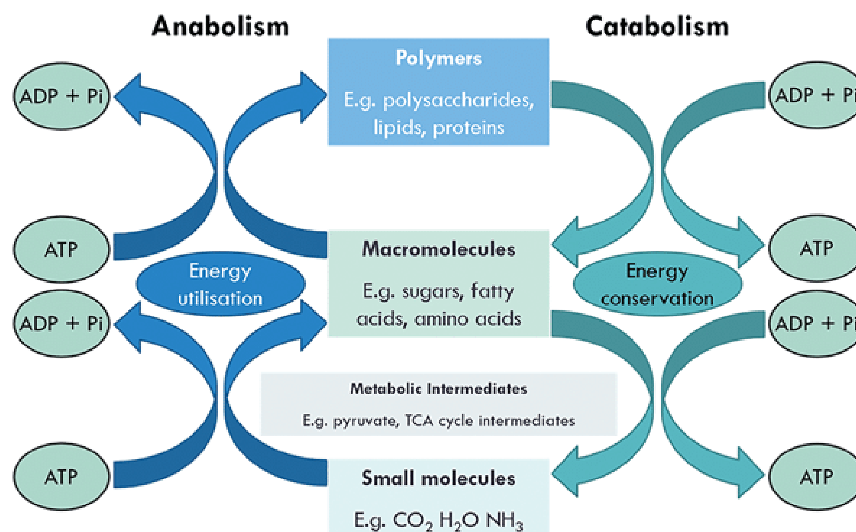

Figure 1: A simplified diagram of anabolic and catabolic reactions with the associated energy transfer [3].

Furthermore, all these molecules take part in different anabolic and catabolic pathways: if the diet, for example, contains a lot of glucose, the organism would stop salvaging their glucose deposits, but prefer glycolysis for energy extraction

and lipogenesis and glycogenesis for storing the excess of glucose. This is only one example of how the body uses and stores nutrients. The metabolism and its allosteric and hormonal regulation are complex and for easier overview will be divided into three main categories: carbohydrate, lipid, and amino acid metabolism.

1.1.1 Carbohydrates

Glucose plays a central role in the mammalian carbohydrate metabolism. Glucose is a monosaccharide mostly found as a part of bigger molecules, like disaccharides (e.g., sucrose) or polysaccharides (e.g., starch). Mammalian cells mostly use glucose for winning energy, which is why the bigger molecules must be broken down to glucose. One possible explanation for this is that glucose has a lower tendency than other aldohexoses to react nonspecifically with the amine groups of proteins [4]. Glucose is ingested in the jejunum with the help of a sodium/glucose cotransporter called SGLT1 (sodium-glucose linked transporter) on the apical membrane. Glucose is then either used for the metabolism of the intestinal cell or transported to the GLUT2 (glucose transporter) on the basolateral membrane so glucose can be transported into the bloodstream. Fructose, on the other hand, has its own apical GLUT5 transporter.

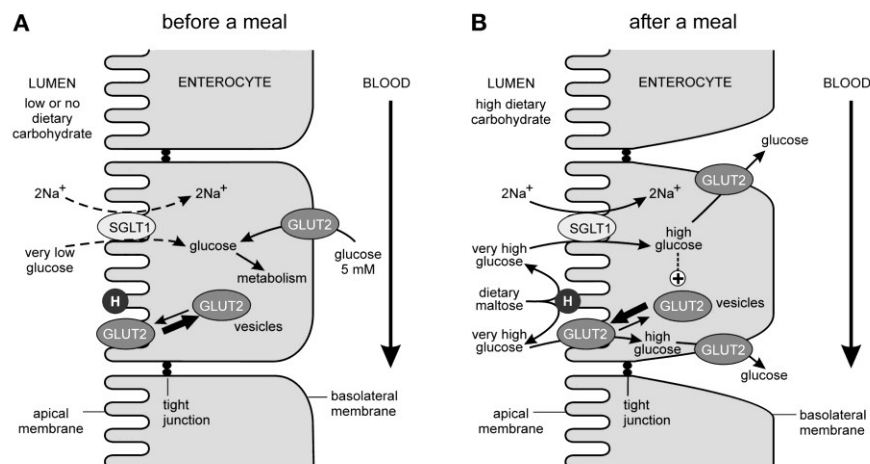

Figure 2: Glucose intake by the small intestine (adapted from George L. Kellett) [5].

Different tissues have different glucose transporters on their cell membranes. Some are controlled by insulin levels, others by glucose concentration. Hepatocytes carry GLUT2 transporters, which act like glucose sensors. The liver also has a specific hexokinase (hexokinase IV, or glucokinase) in the glycolytic pathway with different kinetics. That way, glucokinase (GK) in the liver reaches its

maximum enzyme activity at very high glucose levels. At fasting glucose levels, glucokinase has a very low activity, leaving more glucose for other glucose dependent tissues (for example muscle or brain) and their hexokinases with a low Km (reaches maximum enzyme activity at low glucose levels).

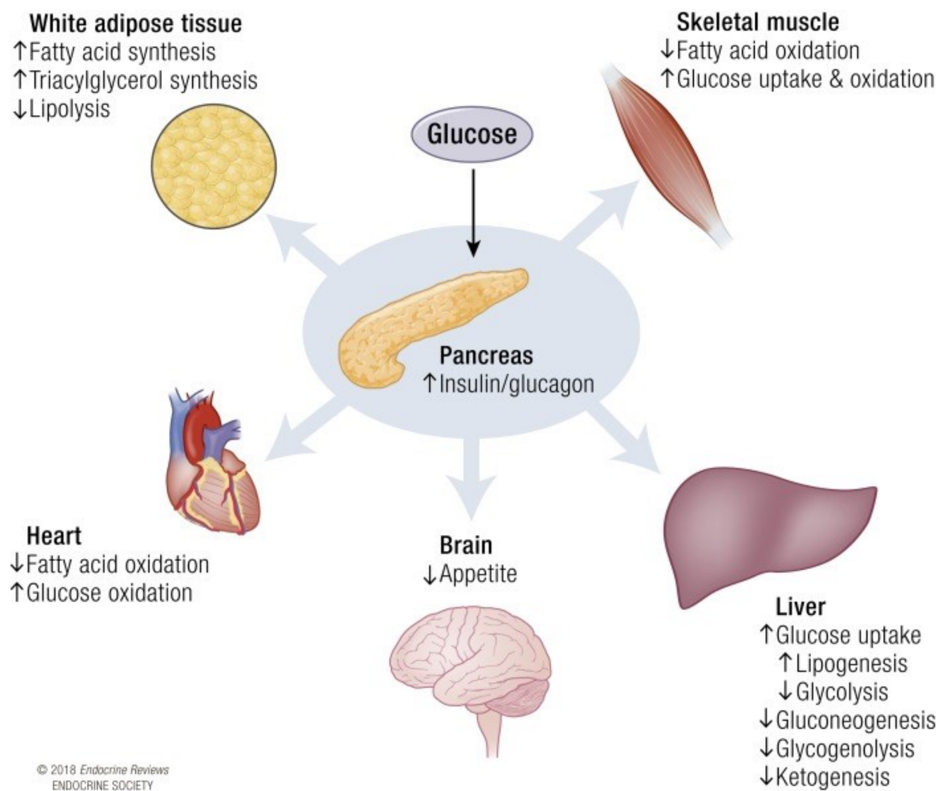

Figure 3: Glucose intake leads to an increase of the insulin/glucagon ratio, which has several effects on different tissues [2].

Glucose can go different ways, depending on the state the organism is at. The common pathways are glycolysis, glycogen metabolism and pentose phosphate pathway.

Glycolysis happens in the cytosol of every cell of an organism and is one of the easiest ways of producing energy. In glycolysis, glucose is sacrificed for producing energy in the form of ATP (net of 2 ATP molecules for 1 glucose molecule), $NADH/H^+$ (a hydrogen transporter important for biotransformation in the liver and peroxide elimination in erythrocytes) and pyruvate, which has different behaviors depending on the oxygen supply of the cell. In the presence of enough oxygen, pyruvate is used for producing acetyl-CoA, which is a key molecule in the intermediary metabolism as it is required for fatty acid and cholesterol synthesis. In

anaerobic conditions, pyruvate is transformed into lactate. Lactate originating from anaerobic glycolysis in the muscle can be transformed back to glucose through gluconeogenesis in the liver. This process is called the Cori cycle. Glycolysis is regulated allosterically and hormonally (through insulin and glucagon). Allosteric regulation of glycolysis includes the negative feedback loop through high ATP, $NADH/H^+$ or citrate levels or regulation through the three key enzymes of glycolysis: hexokinase/glucokinase (HK/GK), phosphofructokinase (PFK) and pyruvate kinase. Hexokinase is inhibited by its product glucose-6-phosphate, which means that glycolysis is a tightly controlled pathway. Glucokinase in the liver, on the other hand, is inhibited by fructose-6-phosphate, the next step of glycolysis, because the liver needs glucose-6-phosphate for glycogen synthesis. PFK-1 is the most important regulatory enzyme of glycolysis, as it is exclusive to glycolysis and is the slowest step in the pathway. Fructose-2,6-bisphosphate is the allosteric activator of PFK-1, so it accelerates glycolysis overall. Fructose-2,6-bisphosphate is found in the liver and muscle tissue and is both produced and broken down by the bifunctional enzyme phosphofructokinase 2/fructose-2,6-bisphosphatase. Pyruvate kinase is the third key enzyme of glycolysis and is allosterically inhibited by ATP and alanine and activated by fructose-1,6-bisphosphate. PFK-2/fructose-2,6-bisphosphatase and pyruvate kinase are also hormonally influenced by insulin and glucagon.

Gluconeogenesis has the exact opposite steps of glycolysis and is mainly happening in the liver, kidney, and intestine. Gluconeogenesis in the liver has the function of maintaining blood glucose levels, even at low dietary sugar intake, as the gluconeogenic precursors are non-carbohydrates. Three enzymes are added to gluconeogenesis, compared to glycolysis: glucose-6-phosphatase, fructose-1,6-bisphosphatase, and phosphoenolpyruvate carboxykinase (PCK). The regulation of gluconeogenesis is mediated by glucagon and insulin levels. Insulin can inhibit gluconeogenic enzymes (e.g., PCK) and therefore inhibit glucose production [6].

Because the glucose transport capacity of the blood is limited, the body had to find a way to store glucose. Because glucose is osmotically active, it is stored as glycogen. The process of storing and mobilizing glucose from glycogen is called **glycogen metabolism**. The main two glycogen reservoirs are in the liver and

muscle tissue. The liver stores around 150 g of glycogen which is available for usage to all tissues. In contrast to the liver, muscle tissue stores around 250 g of glycogen, which can only be used by the muscle itself. Glycogen metabolism is regulated allosterically by glucose, ATP, and calcium levels and hormonally by insulin, glucagon, and adrenaline. High glucose, ATP, insulin, and low calcium levels are triggers for glycogen synthesis, while high AMP, calcium, glucagon, and adrenaline levels trigger glycogenolysis.

The pentose phosphate pathway happens in the cytosol of the cell. It consists of two parts, one is oxidative and irreversible, and the other is non-oxidative and reversible. In the oxidative phase, glucose-6-phosphate is oxidized twice and transformed to produce the reducing equivalent NADPH/H^+ and ribose-5-phosphate which is important for nucleotide, cholesterol, steroid hormone, and fatty acid synthesis. Depending on the state of the organism, the pentose phosphate pathway yields NADPH/H^+ by turning ribose-5-phosphate into glucose-6-phosphate so the pathway can begin anew. The non-oxidative part acts like a connection to glycolysis by producing the glycolysis intermediates F6P and G3P (fructose-6-phosphate and glyceraldehyde-3-phosphate). The oxidative part of the pentose phosphate pathway is regulated by $NADP^+$ concentration in the cytosol which activates the key enzyme glucose-6-phosphate dehydrogenase. NADPH/H^+, on the other hand, inhibits the enzyme. The activity of G6PDH (glucose-6-phosphate dehydrogenase) is relatively low in muscle cells, but it increases as the muscle undergoes atrophy, irrelevant of its genesis [7]. Muscle cells do not need a lot of NADPH/H^+, but they need ribose. Because of this, muscle cells produce ribose from glycolysis intermediates (F6P and G3P) and bypass the NADPH/H^+ production.

1.1.2 Lipids

Although carbohydrates present the fastest way of getting energy in the cell, the highest amount of energy per gram is won through lipids. Aside from this, lipids are important functional membrane molecules. Unlike glucose, lipids are rarely used as the primary energy source. The heart, liver, and muscle, however, extensively use fatty acids (FAs) for winning energy. FAs play a central role in the lipid energy metabolism. During fasting or under long-term exercise, FAs are

broken down to win energy, while glucose is reserved for the glucose dependent cells – erythrocytes and brain cells, where the brain can also use ketone bodies from the excessive breakdown of fatty acids and ketogenic amino acids.
Because of their lipophilic character, lipids are transported in the blood by lipoproteins.

The **breakdown of fatty acids** or **beta oxidation** happens in the mitochondrial matrix. FAs in the cytosol cannot pass the mitochondrial membrane and because of that they must be activated first. This process costs 2 ATP and the result is acyl-CoA. The acyl group is then transferred onto carnitine through carnitine-acyl transferase I (also known as carnitine palmitoyltransferase I or CPT I) making acylcarnitine. The CPT I is inhibited by malonyl-CoA, which also promotes fatty acid synthesis. Acylcarnitine is transported into the mitochondria by the carnitine-acylcarnitine translocase (CACT), and, at the same time, one mitochondrial carnitine is transported into the cytosol. The acyl group is then transferred to a mitochondrial CoA by the CPT II, and the carnitine is left over for a future shift into the cytosol by the CACT.

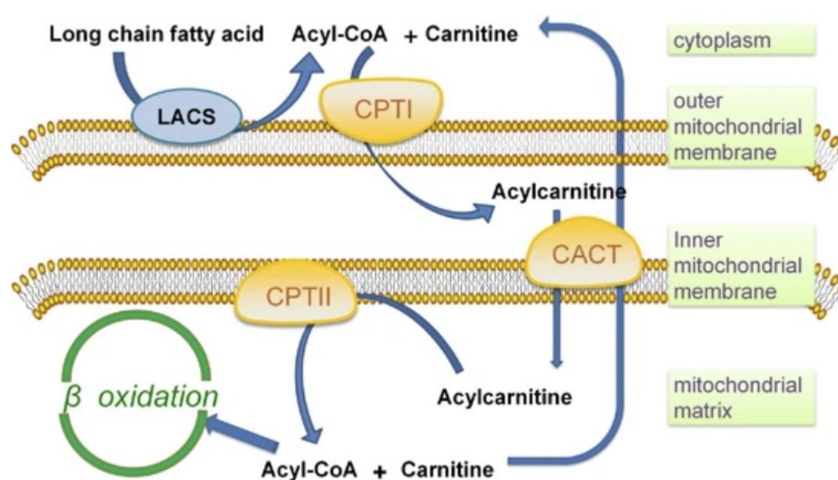

Figure 4: A graphical overview of the role of CPT I, CACT and CPT II in the translocation of fatty acids into mitochondria and beta-oxidation [8].

The fatty acid oxidation cycle consists of four reactions where the fatty acid is shortened by two carbon atoms in each cycle until the FA is completely broken down. One cycle yields one $FADH_2$, one $NADH/H^+$, which both are used in the electron transport chain (ETC), and one acetyl-CoA, which is used in the TCA (tricarboxylic acid) cycle. In the last step of beta oxidation of even numbered fatty

acids, acetoacetyl-CoA is either broken down into two acetyl-CoA molecules or used for ketogenesis. Every acetyl-CoA yields 10 ATP through the TCA cycle and the additional $FADH_2$ and $NADH/H^+$ yield 1.5 and 2.5 ATP molecules respectively. This means that, for example, one palmitic acid, consisting of 16 carbon atoms, yields net of 106 ATP. Beta oxidation is regulated by blood glucose levels – high glucose concentrations inhibit beta oxidation and the activated acyl-CoA forms triglycerides or phospholipids instead of entering the mitochondria. At fasting glucose levels, however, acyl-CoA is transferred into the mitochondria for oxidation. Another regulatory mechanism is the aforementioned malonyl-CoA, which inhibits CPT I allosterically and promotes fatty acid synthesis by fatty acid synthase (FAS).

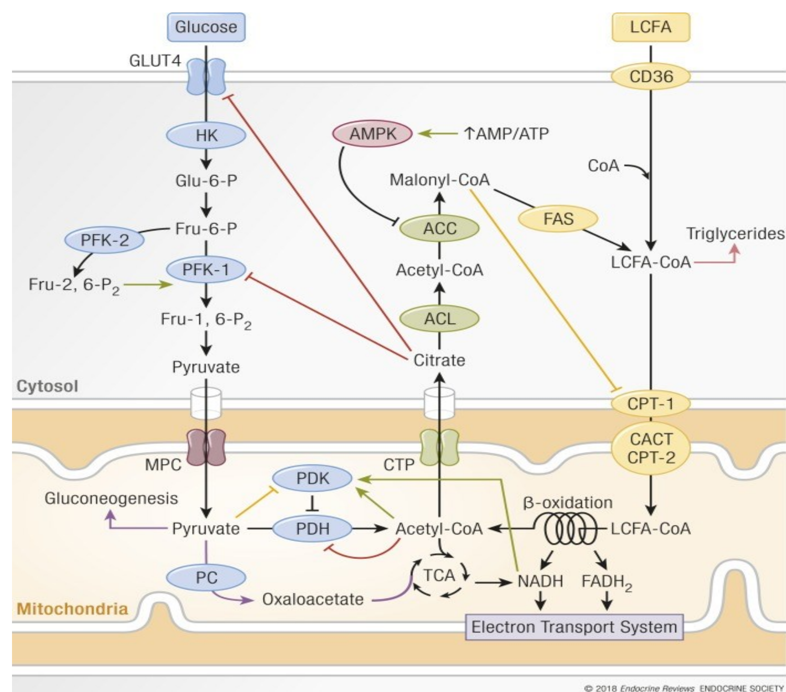

Figure 5: Glycolysis and fatty acid metabolism; malonyl-CoA regulation of CPT I [2].

Excessive dietary carbohydrate or amino acid intake leads to their breakdown for lipid synthesis. At fasting, acetoacetyl-CoA, which is won by beta oxidation of even numbered FAs, is used for ketone body synthesis.

Ketone bodies are important for supplying the brain with energy in longer fasting periods. Another trigger for ketogenesis is diabetes. Ketone bodies are synthesized from accumulated acetyl-CoA or acetoacetyl-CoA from beta oxidation in the mitochondria of hepatocytes. The conversion of ketone bodies back to

acetyl-CoA happens in the mitochondria of extrahepatic cells. Ketone bodies can inhibit gluconeogenesis from certain amino acids (alanine) and therefore slow down the breakdown of proteins.

Fatty acid synthesis happens in the cytosol of every cell type but is most prevalent in hepatocytes of the liver. Moreover, fatty acid synthesis in the brain is of very high importance because these molecules cannot pass the blood-brain barrier. The starting molecule is acetyl-CoA, originating from glycolysis or degradation of amino acids (for example leucine, isoleucine, or tryptophan), but not from beta oxidation, which would be counterproductive. However, acetyl-CoA is made in the mitochondria, so it needs to get into the cytosol for fatty acid synthesis. Because of this, acetyl-CoA must be transformed into citrate, which can pass the mitochondrial membrane. Citrate synthase catalyzes the reaction of oxaloacetate and acetyl-CoA to build citrate, which then enters the cytosol. This is where the enzyme ATP citrate lyase (ACLY) plays an important role. It converts citrate back to oxaloacetate and acetyl-CoA in the cytosol, at the cost of one ATP. It has been shown that a high fat diet can suppress the activity of ACLY, but also acetyl-CoA synthetase (ACSS2) and fatty acid synthase (FASN) in white adipose tissue [9]. The next step is activating acetyl-CoA, by transforming it to malonyl-CoA through acetyl-CoA carboxylase, so it can react with another acetyl-CoA to build fatty acids. This is an important regulatory step in fatty acid synthesis. After this, the fatty acid synthase begins fatty acid synthesis. Allosteric regulation of fatty acid synthesis is mainly through acetyl-CoA carboxylase. It is activated through cytosolic citrate, which also inhibits PFK-1 to slow down production of more citrate in glycolysis. It is inhibited by AMP, signaling a low energy supply in the cell, and by acyl-CoA, signaling beta oxidation. On a hormonal level, adrenaline and glucagon inhibit and insulin activates acetyl-CoA carboxylase. Insulin also activates Acly gene expression and induces the transcription of Fasn mRNA. Another important step is mediated by pyruvate dehydrogenase (PDH) which transforms pyruvate from glycolysis to acetyl-CoA. PDH is activated directly by insulin and additionally by the rising glucose levels, which drive glycolysis, resulting in more pyruvate and acetyl-CoA for fatty acid synthesis. Fatty acids are mainly stored in the adipose tissue as triglycerides (TAG).

Lipogenesis (TAG synthesis) takes place in the adipose tissue, but also in the liver. This is why an imbalance of TAGs in the liver, caused by an increased synthesis and uptake from the bloodstream that is augmented by a reduced output (reduced beta oxidation and VLDL transport), can lead to a fatty liver and later fibrosis and hepatocellular carcinoma [10, 11]. In the process of lipogenesis, three activated FAs bind to one activated glycerol to build one TAG. Fatty acids are activated in the adipose tissue by acyl-CoA-synthetase and ATP, forming acyl-CoAs. Glycerol, on the other hand, is activated through phosphorylation forming glycerol-3-phosphate. The liver and some other organs (kidney, intestine, and mamma) can directly phosphorylate glycerol through glycerol kinase. Glycerol kinase is not present in white adipose tissue, because it would lead to direct TAG synthesis after lipolysis. Glycerol-3-phosphate then, with the help of glycerol-3-phosphate acyltransferase (GPAT), binds to an activated FA (acyl-CoA) to form lysophosphatidic acid. The second acyl-CoA is added with the help of another acyltransferase to form phosphatidic acid, which is subsequently converted into diacylglycerol (DAG) with the help of phosphatidic acid phosphatase (PAP). The third and last acyl-CoA is finally added with the help of diacylglycerol acyltransferase (DGAT) to form triacylglycerol/triglycerides (TAG). Triglycerides are stored in adipose tissue, whereas in the liver it is transformed into VLDL for supplying other tissues with lipids.

Lipolysis (TAG breakdown) primarily takes place in the adipose tissue and intestine. Each of these tissues has their own lipase, which catalyze the hydrolysis of TAGs but only the triacylglycerol-lipase of the adipose tissue is regulated by glucagon and adrenaline. The activity of the lipase in the adipose tissue can therefore increase when the organism is fasting so it can break more TAGs down for energy. The end products of lipolysis (glycerol and three FAs) are used in other tissues for beta oxidation or glycolysis. Glycerol is also a substrate for gluconeogenesis.

In general, fasting leads to increased lipolysis, while decreasing *de novo* lipogenesis (DNL). Contrary, overfeeding would lead to downregulation of lipolysis and promote *de novo* lipogenesis instead. It has been shown that intermittent fasting inhibits *de novo* lipogenesis in the liver, which could prove beneficial for treating hepatic steatosis [12]. Furthermore, a high-fat-diet has shown to promote

hepatic steatosis, while decreasing *de novo* lipogenesis, which suggests that the cause of a fatty liver during this diet are the existing fats or the dietary fat itself [13]. There are more lipids and derivatives of lipids, like phospholipids and glycolipids, which are important components of cell membranes and can act like transmitters. Cholesterol, which is another type of lipid and an important laboratory marker, will be discussed in a separate chapter.

1.1.3 Cholesterol and Bile Acids

Cholesterol is another type of lipid, which are called sterols. Cholesterol is an important part of the cell membrane but is also used in cell signaling and bile acid, steroid hormone, and vitamin D synthesis.

Cholesterol biosynthesis starts in the cytosol and endoplasmic reticulum of every cell, but especially so in the liver and intestine. Most of the cholesterol in the body comes from intrinsic synthesis, whereas just a small amount from dietary intake. Cholesterol is a steroid consisting of 27 carbon atoms, which are derived from acetyl-CoA. Acetyl-CoA and acetoacetyl-CoA are bound by HMG-CoA synthase to form HMG-CoA. HMG-CoA is then reduced to mevalonate by HMG-CoA reductase. HMG-CoA reductase is kinetically the most important enzyme of cholesterol synthesis. From there, mevalonate is further processed to isopentenyl pyrophosphate. The next steps are forming geranyl and later farnesyl pyrophosphate. Farnesyl pyrophosphate can be also used for dolichol and ubiquinone synthesis and protein prenylation. Cholesterol biosynthesis is controlled allosterically by mevalonate and cholesterol and hormonally by insulin and glucagon [14]. An excessive amount of cholesterol in the cell can be stored in the cytosol as cholesteryl esters. The esterification is done by the transferases ACAT (sterol O-acyltransferase) and LCAT (lecithin-cholesterol acyltransferase).

Both the white and brown adipose tissues (WAT and BAT) are lipid and cholesterol storages, which made them interesting research targets regarding their role in metabolic diseases. It has been shown that the activation of BAT through cold adaptation or β3-adrenergic receptor agonists leads to increased lipoprotein flux and decreased serum cholesterol and triglyceride levels [15, 16].

Bile acids are steroid acids and their salts play an important role in the digestion of dietary fats by emulsifying them. Bile acids also play an important role in the

uptake of fat-soluble vitamins, cholesterol elimination and cell signaling by binding to different receptors, like the farnesoid X receptor (FXR).
Primary **bile acid synthesis** happens in the hepatocytes, where cholic and chenodeoxycholic acids (CA and CDCA, respectively) are synthesized from cholesterol. Because this diploma thesis revolves around research done in mice, it is worth noting that bile acids in mice are somewhat different than in humans and some of them are murine-specific (e.g., muricholic acids). The rate-limiting step is also the first step of classical bile acid synthesis, which is catalyzed by cholesterol 7 alpha-hydroxylase (CYP7A1). Bile acids are then conjugated by either taurine or glycine into their conjugated forms (salts) to increase their solubility in water before they are released into the duodenum to fulfill their function. Bile salts are then modified by the bacteria in the intestine or deconjugated into secondary bile acids (secondary bile acid synthesis). Bile acids are either reabsorbed into the enterohepatic circulation (about 95% of the bile acids) or are excreted.

1.1.4 Proteins and Amino Acids

Proteins are amino acid chains, which are coded by DNA/RNA and being synthesized by every cell in the body. The enzymes, which have been mentioned earlier, are in fact proteins (enzymes). Protein synthesis is a complex process, which is regulated and influenced by a lot of factors. Glucose and insulin can have different effects on protein synthesis depending on the tissue. For example, glucose increases the rate of protein synthesis in pancreatic β-cells and skeletal muscle cells, whereas this effect could not be shown in the duodenum [17, 18]. Furthermore, both insulin and glucose have shown to increase protein synthesis in the muscle and insulin decreases protein catabolism in the liver and adipose tissue [19, 20]. Interestingly, a high-fat-diet and a diet rich in cholesterol have shown to inhibit hepatic protein synthesis in rats [21].
After translation, the protein needs to undergo post-translational processing to gain its function. This is done by glycosylation, controlled proteolysis, and amino acid modifications. Some proteins (e.g., key enzymes in metabolic pathways) have a short half-life, while others (e.g., lactate-dehydrogenase) have a longer half-life. Either way, cellular proteins are eventually degraded by proteasomes or lysosomes in the cell. Because there is no special amino acid or protein depot in the body, amino acids needed for protein biosynthesis are either being taken in by

food, synthesized in the cells or salvaged from other tissues. This is also the reason excess amino acids undergo rapid catabolism [22]. There are 22 proteinogenic amino acids, which are incorporated into proteins. Some of these are called functional amino acids, which are involved in cell signaling and gene expression [23].

Because amino acids are so valuable, the body has found a way to convert amino acids one into another. This process is called transamination, which is catalyzed by aminotransferases and happens mainly in the liver. Although every cell can synthesize amino acids, the liver plays the biggest part in amino acid synthesis. Essential amino acids are amino acids that cannot be synthesized by the cells and must be taken in through the diet. It has been shown that branched-chain amino acids (BCAA) are the most important contributors to the effects of dietary protein supply on metabolic health and body fat [24]. The synthesis of non-essential amino acid is connected to several pathways, including the TCA cycle, glycolysis, pentose phosphate pathway and urea cycle.

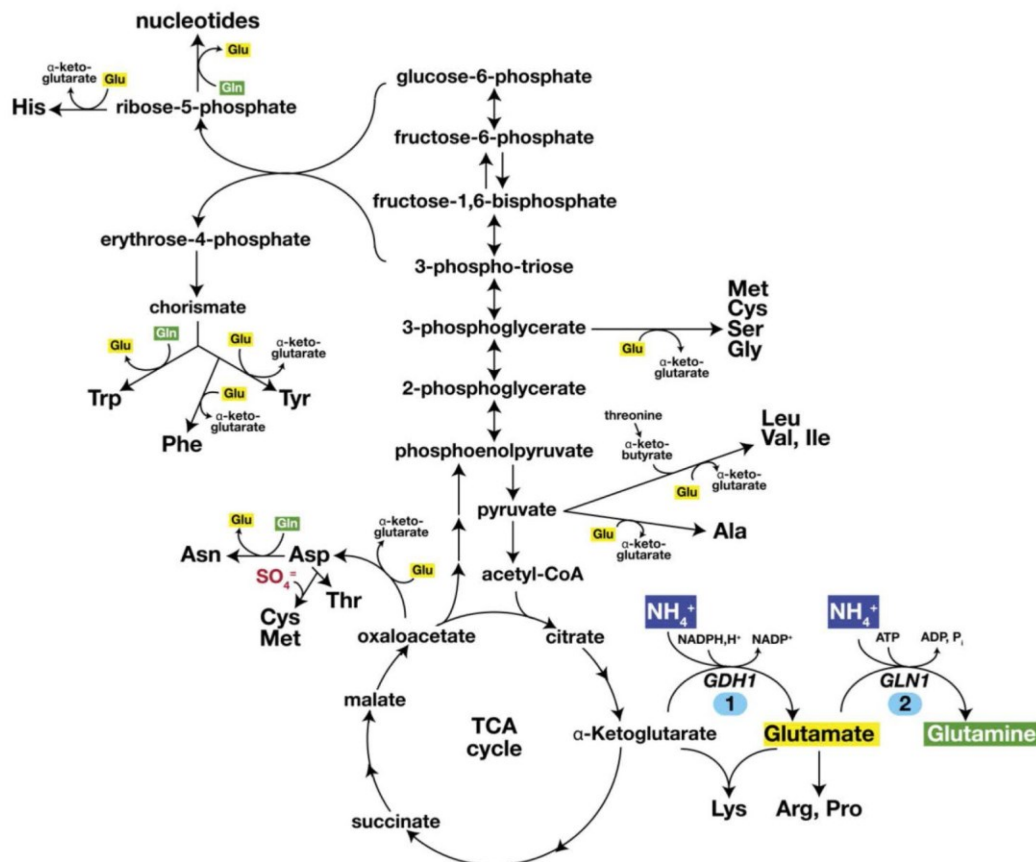

Figure 6: An overview of amino acid synthesis (adapted from P. Ljungdahl) [25].

The breakdown (digestion) of dietary protein into amino acids begins in the gastrointestinal tract and is achieved by different peptidases. Excessive protein

intake can lead to amino acid overflow, where the amount of dietary amino acids surpasses the metabolic needs of the organism (e.g., protein synthesis). Excess amino acids undergo increased oxidative deamination of their amino groups to produce ketone bodies or gluconeogenesis to produce glucose [26]. Oxidative deamination happens in the liver, while the catabolism of the carbon skeleton happens in every cell with mitochondria. The toxic byproduct of oxidative deamination is ammonia, which is directly neutralized into urea through the urea cycle in the liver and then excreted by the kidney. An important amino acid for gluconeogenesis in fasting is alanine, which originates from muscle protein. Amino acids are divided into glucogenic and ketogenic amino acids. Glucogenic amino acids can be converted into glucose through gluconeogenesis, while ketogenic amino acids can be converted into ketone bodies, used for fatty acid synthesis, or degraded in the TCA cycle [22]. Most amino acids are glucogenic, some are both glucogenic and ketogenic, while leucine and lysine are only ketogenic.

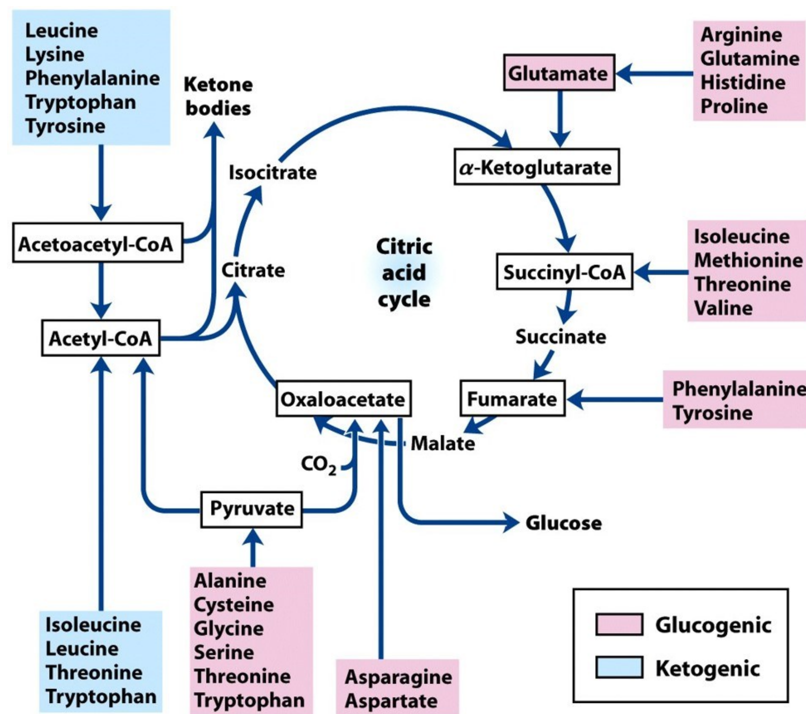

Figure 7: An overview of amino acid conversion and integration into the TCA cycle [27].

1.2 Gene Transcription and RNA Processing

You are what you eat – a common proverbial saying that is probably used on a daily basis and is used as a motto by nutritionists when talking about a healthy diet. But how true is this saying, how can we become what we eat, how can our diet change our body? Studies have shown that our diet can indeed change the way our cells behave and even change our organism on a bigger scale. It has been shown that dietary and nutritional restriction can alter physiological processes and survival of certain organisms. Furthermore, dietary restriction can help maintaining the youthfulness of the gene regulation and expression [28]. A ketogenic diet alone can increase the lifespan and improve cognition in adult mice [29, 30]. Studies have also shown positive effects of ketogenic diet on neurological diseases [31-33]. These findings inspired many researchers to look for other dietary effects on gene regulation and health. The genome of every cell is under control of complex and diverse factors that influence the transcriptional, post-transcriptional, translational, and post-translational regulation of genes. The focus in this research lies in the transcriptional gene regulation. The first step in protein biosynthesis is the transcription of the DNA in the nucleus to produce a precursor RNA (pre-mRNA) molecule which includes coding, but also non-coding regions of a gene (called exons and introns, respectively).

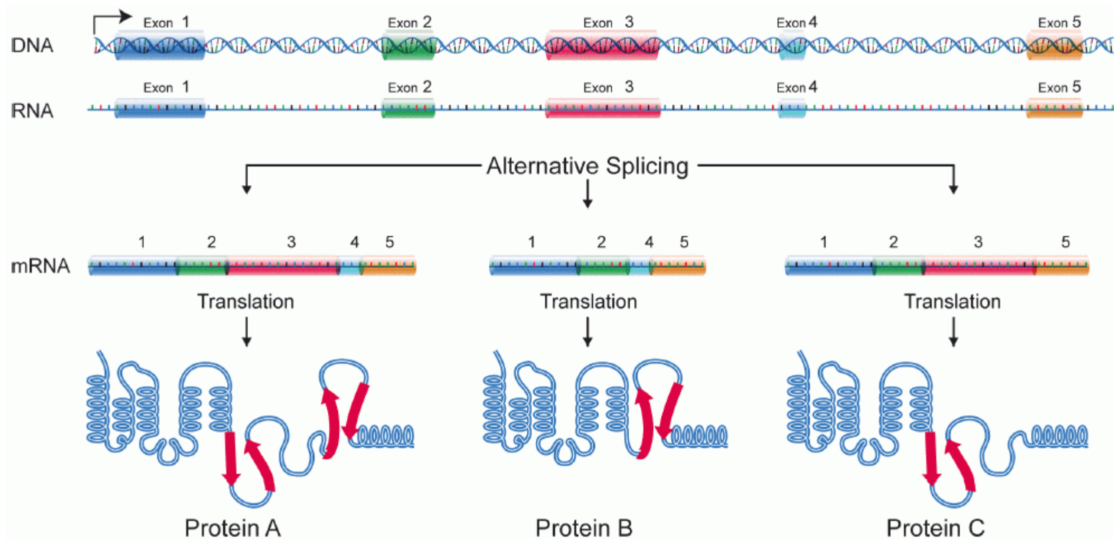

Figure 8: A graphical overview of transcription and translation in protein biosynthesis (adapted from National Human Genome Research Institute) [34].

The pre-mRNA is spliced by the spliceosome into mature mRNA molecules. The mature mRNA carrying the transcribed heterocyclic base sequence then travels

into the cytoplasm to bind to a ribosome, where transfer RNAs (tRNAs) will bind in a codon-anticodon pattern to produce the encoded amino acid sequence. One tRNA has two important sites, one carries the anticodon sequence - a triplet of heterocyclic bases that correspond to a codon on the mRNA, and the other carries the amino acid. The pre-mRNAs of certain genes undergo alternative splicing events which can lead to different mRNAs and to the translation of proteins with slightly changed structure, thus also having different properties than the other variants of that protein. This is one example for protein isoforms – proteins with a similar amino acid sequence and function that are produced by different genes or the same gene through alternative splicing.

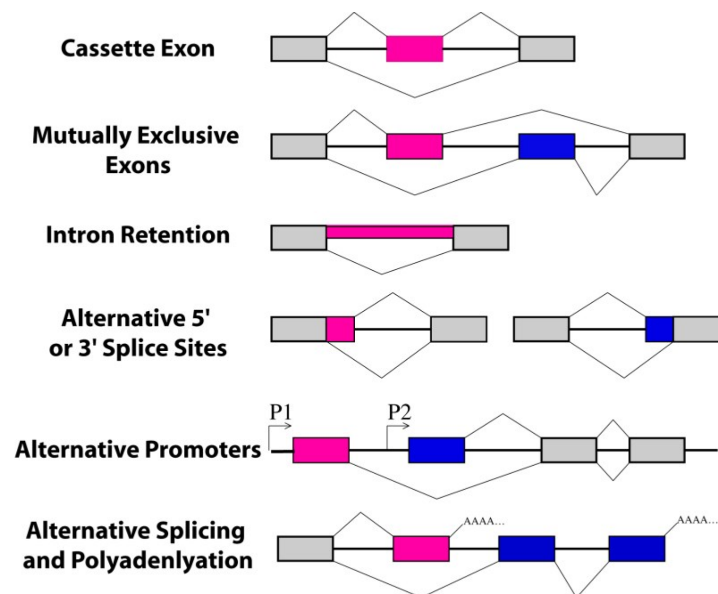

Figure 9: Schematic representation of different RNA processing mechanisms.
The emphasis of the conducted investigation lies in alternative splicing and alternative promoters (adapted from Alan M. Zahler) [35].

Global pre-mRNA splicing is crucial for gene expression and its linkage to the proteome of an organism. Around a half of human genes show alternative splicing events [36-39]. Alternative splicing is also important for tissue-specific expression of transcript isoforms [40-44]. It has been shown that dietary restriction can reduce the defects in global pre-mRNA splicing of aging mice in comparison to animals that were fed ad libitum [28]. Deregulation of splicing events leads to multiple age-related diseases. Thus, alternative splicing plays an important role in health and can be influenced by dietary behavior. In this diploma thesis, changes in the regulation of alternative splicing events and alternative promoter usage of certain

genes were investigated by measuring the amount of the corresponding alternative mRNAs (isoforms) in mice after feeding special diets.

1.3 Aim of the Study

The background of this study lies in the previous investigations of dietary effects on gene expression, yet many effects are not fully understood. The conducted research in this diploma thesis revolves around the influence of special diets on the expression of certain genes involved in metabolic pathways in murine liver and brown adipose tissue. Most researchers aimed at analyzing gene expression in the liver, while this diploma thesis also aims at exploring the dietary effects on gene expression in the brown adipose tissue, which is also metabolically active. Thus, having comparable results from two different tissues, this study gives insight in the inter-organ relationship of the investigated genes, as well as the possible differences in alternative splicing or alternative promoter usage between the tissues caused solely by changes in dietary regimes. Furthermore, some of the investigated genes play important roles in disease development, so the results could prove useful in the clinical setting. It is worth mentioning that gene expression as well as the effects of these diets might differ in humans, so the data in this diploma thesis should be further tested and adapted to humans.

2 Material and Methods

2.1 Mice and Diets Used

Five different diets in comparison to standard rodent chow diet (rich in carbohydrates) were used to feed C57BL6 wild type mice. One group of mice was fed a protein free diet for 4 weeks, the next one was fed a high fat diet for 4 weeks, another group were fed a ketogenic diet for 4 weeks and the fourth group were fed a western diet (1.25% cholesterol = 12.950 mg/kg). Knockout models were not used, in order to represent the mere effects of diets on the gene transcription and mRNA processing.

Composition of the Diets				
	Protein-Free-Diet	**High-Fat-Diet**	**Ketogenic Diet**	**Western Diet (1.25% cholesterol)**
Crude Protein	< 0.1 %	24.1%	8.0%	17.3 %
Crude Fat	8.0%	34.0%	79.2%	21.1%
Sugar	14.1%	23.8%	0.7%	34.1%
Starch	38.5%	1.1%	0.6%	13.4%
Crude Fiber	12.0%	6.0%	5.0%	5.0%
Crude Ash	5.2%	6.0%	4.5%	4.2%
Vitamin A	15,000 IU/kg	15,000 IU/kg	15,000 IU/kg	15,000 IU/kg
Vitamin C	30 mg/kg	30 mg/kg	30 mg/kg	30 mg/kg
Vitamin D3	1,500 IU/kg	1,500 IU/kg	1,500 IU/kg	1,500 IU/kg
Vitamin E	150 mg/kg	150 mg/kg	150 mg/kg	150 mg/kg
Vitamin K3	20 mg/kg	20 mg/kg	20 mg/kg	20 mg/kg
Copper	13 mg/kg	12 mg/kg	11 mg/kg	11 mg/kg

Table 1: Composition of the diets used.
Protein-free-diet (PFD), high-fat-diet (HFD), ketogenic diet (KD), western diet (WD). The base nutrients differ significantly, while the amount of vitamins and minerals remained the same across all diets.

As shown in the table, the diets differ significantly in their amount of protein, fat and carbohydrates, whereas the amount of other nutrients remained roughly the same.

2.2 RNA Isolation

Samples were homogenized and disrupted with 600 µl Trizol® Reagent in combination with ceramic beads using a MagnaLyser centrifuge. The centrifuge rotates and tilts the samples 2 times 20 seconds, so the tissues and the reagent completely homogenize. At the following step 120 µl (1/5 of the Trizol used) of chloroform (Emsure) was added and shaken well. Moreover, the samples were put

into the centrifuge (Heraeus™ Fresco 17) at 13000 rpm at 4 degrees Celsius for 20 minutes to separate the mixture into three phases: on the bottom the low phase with proteins, the intermediate phase with mainly DNA and the upper aqua phase that contains the RNA. The same rotation speed and temperature were used for all centrifuge runs. The aqueous phase was then pipetted into another tube and the rest was discarded.

On the following step 500 µl Isopropanol (Emsure) were added to precipitate the RNA, then centrifuged at 13000 rpm for 20 minutes at 4 degrees Celsius. The supernatant was removed afterwards. The precipitate was washed with 500 µl of 70% Ethanol. After proper shaking, the whole tube was centrifuged again for 20 minutes, and the supernatant was thrown away. The tube was left to dry. The RNA was diluted according to the pellet size with distilled water (Fresenius H_2O). Then the tubes were put for 10 minutes on 65 degrees Celsius and at last put into the freezer.

To determine the RNA concentration, the NanoDrop™ 2000 spectrophotometer with a 280/260 ratio and a 260/230 ratio was used to check if the samples were clean enough for cDNA synthesis.

2.3 cDNA Synthesis

A mastermix consisting of 4 µl 5x Buffer, 2 µl 0.1 M DTT (dithiothreitol), 0.3 µl dNTP (deoxynucleotide triphosphate mix), 0.5 µl Superscript, 0.5 µl RNAse Inhibitor, 0.4 µl Random Hexamers was mixed together. Using a pipette, 7.5 µl of the mastermix was mixed with 1 µg RNA of our samples and distilled water to a total volume of 15 µl. Our samples and mastermix were then put into the My Cycler® Thermal Cycler machine to synthesize cDNA. The first step lasting 90 minutes at 42 °C was followed by the second step lasting 15 minutes at 70 °C followed by a 4 °C stage of indefinite duration. A negative control, consisting of the sample and mastermix but without the superscript, was used. After that, the cDNA was given into PCR tubes and diluted with 190 µl of distilled water. The rest of the cDNA was given into a tube to form a pool. From that pool, the cDNA was diluted to five standards (1:5, 1:10, 1:20, 1:40 and 1:160).

2.4 qPCR

Quantitative PCR was used for determining the amount of transcribed mRNA of the investigated genes. First, the RNA was isolated from the tissue, followed by complementary DNA synthesis, so it can be measured with qPCR. To analyze gene transcription, 2 µl of cDNA and 8 µl of mastermix (New England Biolabs) were put into well plates as duplets and analyzed with Light Cycler 480. The mastermix was made of 5 µl SYBR, 0.5 µl Primers and 2.5 µl water per well. Plates were centrifuged, put into the Light Cycler and passed through a cycle, containing following steps: heating up to 50 degrees Celsius – denaturation at 95 degrees Celsius - amplification and quantification by cycling 40 times at 95°C for 15 seconds and 60°C for one minute, followed by a melting curve analysis by continuous heating and measuring from 55°C to 95°C and Cooling to 40°C. An RNA control (190 µl distilled water and 10 µl RNA) and negative control using only distilled water were used to see if the samples were clean and eligible for PCR analysis. Because the sample size was not big, massive outliers were excluded. The qPCR results of each gene were normalized by comparing the results to a housekeeping gene (Hprt), to get the most accurate results possible.

2.5 Custom Primers

To quantitate the different isoforms and to detect alternative splicing events influenced by the diets, custom primers were designed for genes that had known isoforms but no available isoform-specific primers for qPCR. To achieve that, mRNA sequences from the NCBI (National Center for Biotechnology Information) "Gene" search engine were used to check how many isoforms of that gene are known, and if they can be detected by the qPCR. Then, the sequences of the isoforms were aligned using Clustal Omega Sequence Alignment tool to see where exactly the alternative splicing and/or transcription take place. Using BLAT Search (BLAST-like Alignment Tool), exons affected by the alternative splicing were detected, in order to determine where the custom primers should bind. The primers were tested using BLAST (Basic Local Alignment Search Tool), to check if they are in fact specific for that sequence in the genome. The custom primers were either designed using the GenScript Primer Design tool or were freely chosen according to the sequence of each isoform. These methods were also

used to highlight the presence of alternative promoters in some genes (e.g., Chrebp-α/β) and differences in the pre-mRNA and mature mRNA of other genes (e.g., Cyp7a1).

Custom Primers	
Acly (longer transcript)	FW: *AGATTCAGTCCCAAGTCCAAGATCCCTGCA*
	RV: *GGCTCGTCTCGGGAACACAC*
Pfkm (muscle specific)	FW: *TGTGGTCCGAGTTGGTATCTT*
	RV: *GCACTTCCAATCACTGTGCC*

Table 2: Custom forward and reverse primers used to measure Acly and Pfkm isoform mRNA levels.

The longer Acly transcript was determined and separated from the shorter one, by using a custom forward primer, which binds to Exon 14, which is exclusive to the longer transcript.

3 Results

The genes investigated in this research were chosen considering what is already known and where it was expected to see dietary effects. Because the goal was finding new dietary impact on gene expression and alternative splicing, the investigation involved genes from various metabolic pathways and two tissues – liver and brown adipose tissue (BAT). To accomplish this, mRNA levels of various genes were measured and compared to a control group. There were n=4-6 mice for every diet used and the results were compared to a control group (n=9). The statistical significance was measured using Student’s two-sample t-test (two-tail p-value).

3.1 Genes of the Carbohydrate Metabolism in Liver & BAT

This section provides a brief overview of the investigated genes involved in the carbohydrate metabolism.

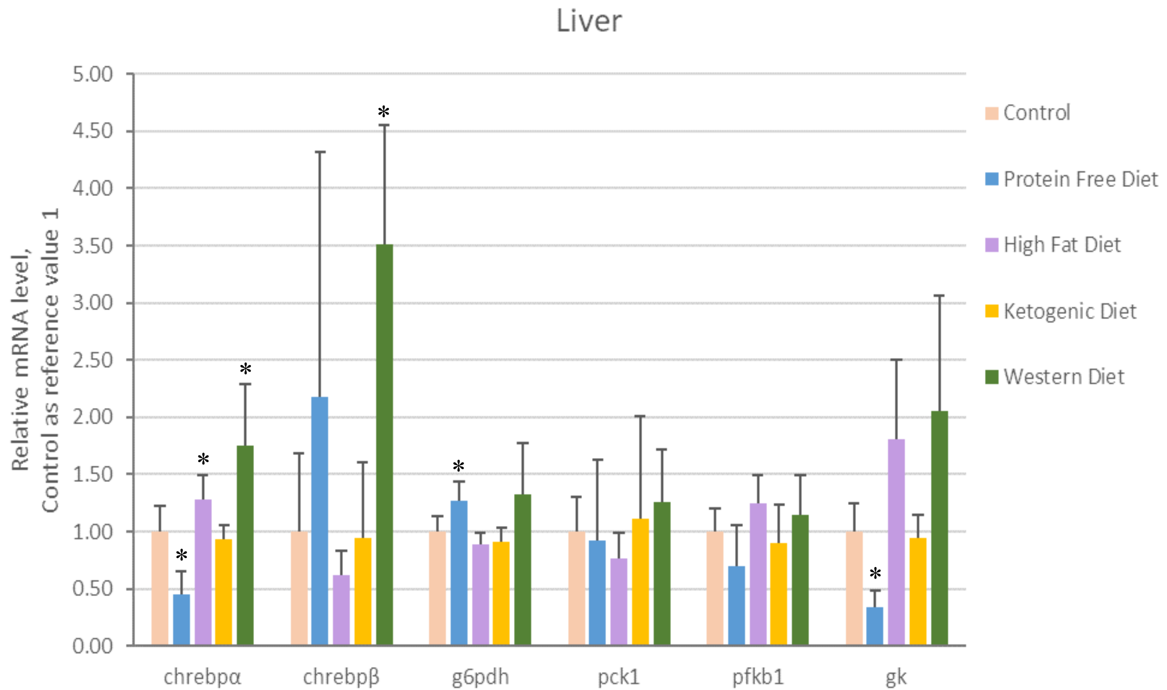

*Figure 10: Overview of relative mRNA level of genes in carbohydrate metabolism in the liver after feeding a protein free, high fat and ketogenic diet for 4 weeks and western diet for 5 weeks. Chrebpα/Chrebpβ – carbohydrate-response element-binding protein (isoforms α and β); G6pdh (G6pdx) – glucose-6-phophat dehydrogenase; Pck1 – phosphoenolpyruvate carboxykinase 1; Pfkb1 – phosphofructokinase; Gk – glucokinase (hexokinase 4). *p<0.05.*

As shown in the chart, the mRNA levels of Chrebp, which encodes a transcription factor that is involved in the development of metabolic syndrome and targets genes in glycolysis, gluconeogenesis and lipogenesis, were highly affected by the diets used [45]. Also, the highly expressed glucokinase was affected by protein dilution, as well as a high fat and western diet. The protein free diet decreased Chrebp-α mRNA level by 55%, while Chrebp-β mRNA level more than doubled, showing a 118% increase. Thus, the shift in relative mRNA levels of these isoforms caused by protein free diet seems to be inversely proportional (with a ratio of 1:4.84). The high fat diet increased Chrebp-α mRNA by 28% and reduced Chrebp-β mRNA by 38%. This correlation also seems to be inversely proportional (ratio 1:2.06). The western diet increased relative mRNA levels of both Chrebp

isoforms, especially isoform β, that showed a 251% increase. Chrebp-α mRNA level was increased by 75% with a western diet. The response of Chrebp to sugars was seen in the increase in mRNA levels with the western and, only in isoform α, with the high fat diet. The mRNA levels of G6pdh and Pck1 were not considerably influenced by the diets used. Both Pfkb1 and Gk mRNA levels decreased by 30 and 67% respectively by the protein diet, while the high fat and western diet almost doubled Gk mRNA level (+81% for HFD and +106% for WD). The ketogenic diet had no considerable impact on the mRNA levels of these six genes in the liver across all diets used. Hexokinase 2 and Pfkm (muscle type isoform) were also investigated but showed no expression in the liver. Overall, the high fat and western diet were similar in their effects on mRNA levels, whereas the protein free diet seemed to have the least predictable results.

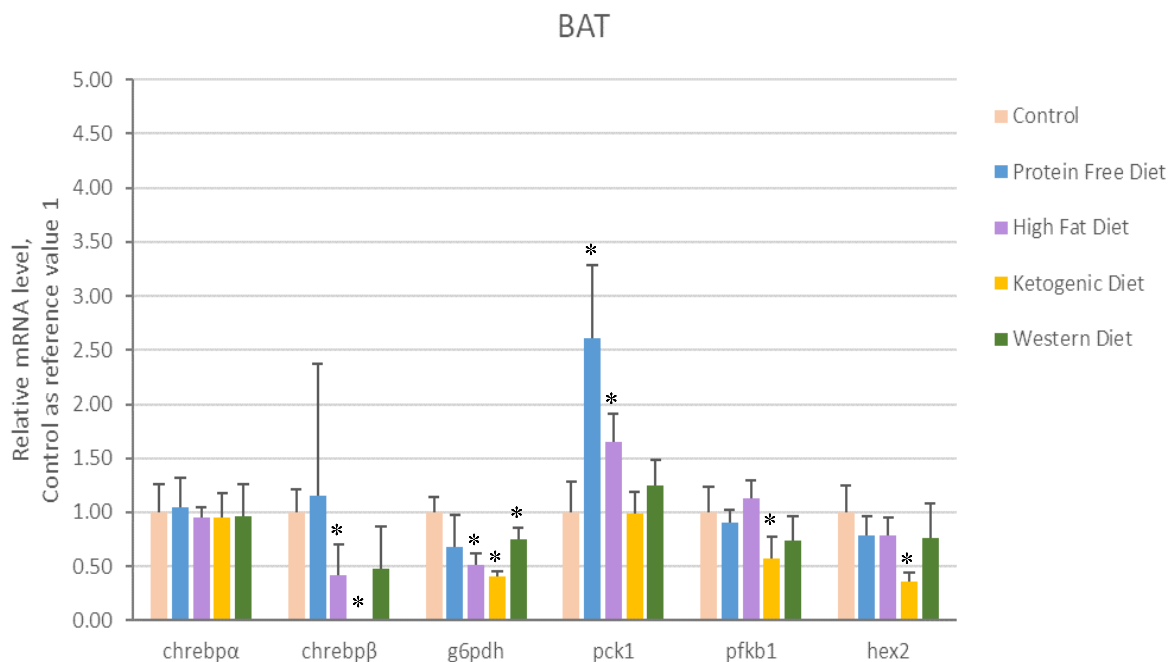

Figure 11: Overview of relative mRNA level of genes in carbohydrate metabolism in brown adipose tissue after feeding a protein free, high fat and ketogenic diet for 4 weeks and western diet for 5 weeks.

*Chrebpα/Chrebpβ – carbohydrate-response element-binding protein (isoforms α and β); G6pdh (G6pdx) – glucose-6-phophat dehydrogenase; Pck1 – phosphoenolpyruvate carboxykinase 1; Pfkb1 – phosphofructokinase; Hex2 (Hk2) – hexokinase 2. *$p<0.05$.*

In contrast to the changes in Chrebp-α mRNA level in the liver, in BAT there were no dietary effects on the mRNA expression of this gene. Chrebp-β mRNA levels, on the other hand, were halved with the high fat (-58%) and western diet (-53%),

while a ketogenic diet completely suppressed Chrebp-β mRNA expression. The mRNA expression of G6pdh decreased with every diet used, especially so with the ketogenic (-60%) and high fat diet (-48%). The protein free diet increased Pck1 mRNA level dramatically, showing a 161% increase, while a high fat diet led to a 65% increase. Pfkb1 mRNA level was almost halved with the ketogenic diet (-43%), while the western diet decreased mRNA level by 26%. Hexokinase 2 (Hk2) is highly expressed in fat, colon, and testis. Hk2 mRNA level was more than halved with the ketogenic diet (-64%), but also decreased with the protein free (-22%), high fat (-21%) and western diet (-23%).

3.2 Genes of the Lipid Metabolism in Liver & BAT

This section shows an overview of the investigated genes of the lipid metabolism.

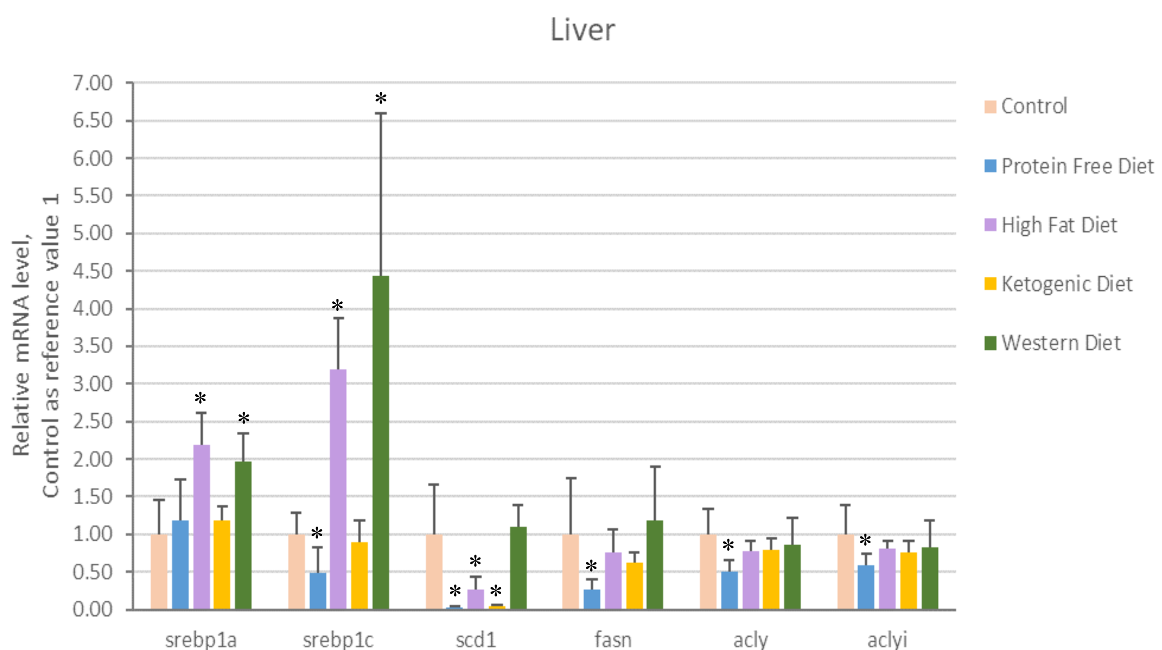

*Figure 12: Overview of relative mRNA level of genes in lipid metabolism in the liver after feeding a protein free, high fat and ketogenic diet for 4 weeks and western diet for 5 weeks. Srebp1a/Srebp1c - sterol regulatory element-binding protein 1 (isoforms a, c); Scd1 - stearoyl-CoA desaturase; Fasn – fatty acid synthase; Acly/Aclyi (ATP citrate lyase and its isoform). *p<0.05.*

The lipid metabolism in the liver was strongly influenced by the diets used. The transcription factor Srebp-1 is an important regulator of fatty acid synthesis and lipogenesis. The relative mRNA level of Srebp-1a was doubled with both the high fat and western diet. More specifically, the high fat diet led to an increase of 119%,

while the western diet increased Srebp-1a mRNA levels by 96%. Contrary, the mRNA level of isoform Srebp-1c was halved with the protein free diet, while the high fat and western diet increased mRNA levels by 220% and 343% respectively. The mRNA level of Scd1, which is the rate-limiting enzyme in monounsaturated fatty acid (MUFA) formation, got completely suppressed with the ketogenic and protein free diet, while the high fat diet decreased mRNA level by 73% [46]. Protein dilution has led to a 74% decrease in Fasn mRNA level and 50% decrease in Acly mRNA level. There was no difference in relative mRNA level between Acly and its isoform.

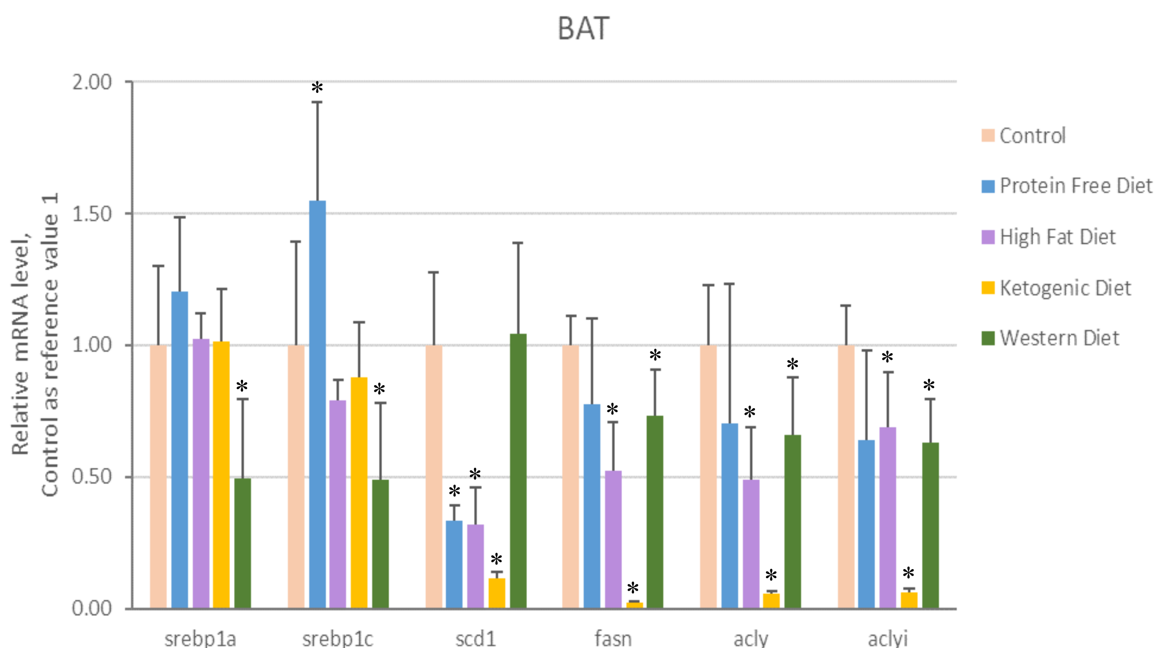

*Figure 13: Overview of relative mRNA level of genes in lipid metabolism in brown adipose tissue after feeding a protein free, high fat and ketogenic diet for 4 weeks and western diet for 5 weeks. Srebp1a/Srebp1c - sterol regulatory element-binding protein 1 (isoforms a and c); Scd1 - stearoyl-CoA desaturase; Fasn – fatty acid synthase; Acly/Aclyi (ATP citrate lyase and its isoform). *p<0.05.*

The relative mRNA levels of Srebp-1a and Srebp-1c were halved with a western diet, with both showing a 51% decrease compared to control. Protein dilution increased relative Srebp-1c mRNA level by 55%, while increasing Srebp-1a mRNA level by only 20%. The high fat and ketogenic diet decreased Srebp-1c mRNA level by 21% and 12% respectively compared to control. Overall, the influence of diets on Srebp-1a and Srebp-1c mRNA levels in brown adipose tissue showed an inversely proportional relationship in comparison to the liver. The

ketogenic diet did not considerably change mRNA levels of Srebp-1 in both tissues. Relative Scd1 mRNA levels were suppressed by 67% with a protein free diet, by 68% with a high fat diet, and got almost completely suppressed with a ketogenic diet with an 89% decrease. The ketogenic diet also almost completely suppressed relative Fasn and Acly mRNA levels, showing a 98% and 95% decrease respectively. Protein dilution, as well as the high fat and western diet also suppressed relative Fasn and Acly mRNA levels. Relative Fasn mRNA levels were decreased by 22% with a protein free diet, by 48% with a high fat diet and by 27% with a western diet, while these diets suppressed relative Acly mRNA levels by 30%, 51% and 34% respectively. The mRNA levels of the Acly isoform were not notably different compared to Acly.

3.3 Genes of the Cholesterol and Bile Acid Metabolism in Liver & BAT

This section covers some genes crucial for cholesterol and bile acid metabolism.

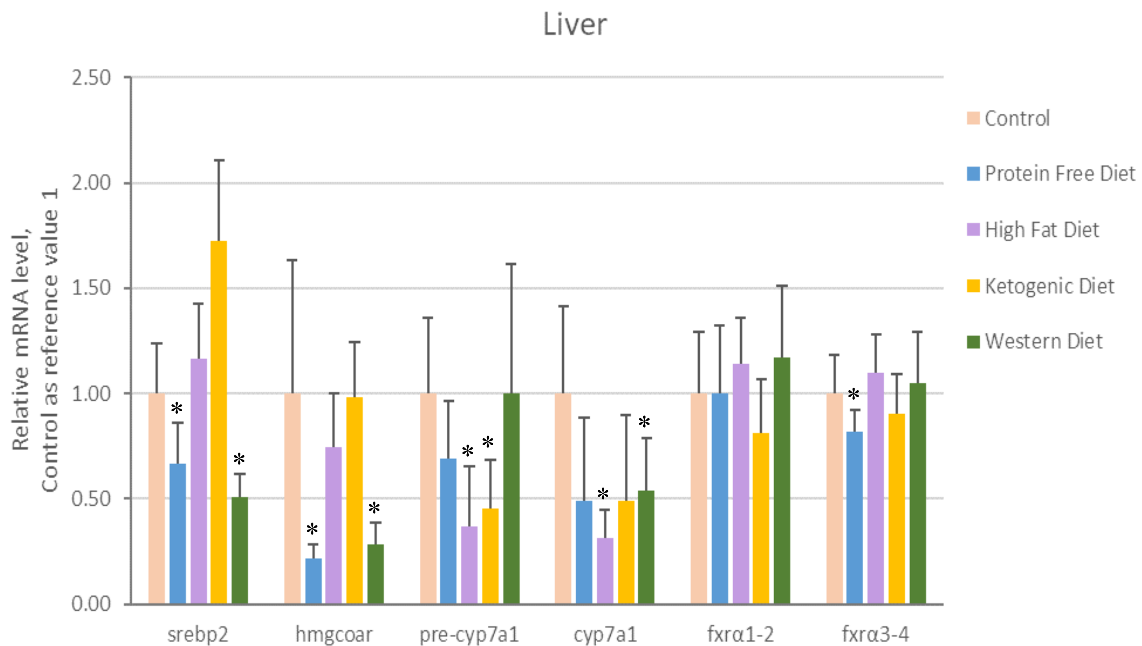

Figure 14: Overview of relative mRNA level of genes in cholesterol and bile acid metabolism in the liver after feeding a protein free, high fat and ketogenic diet for 4 weeks and western diet for 5 weeks.

*Srebp2 - sterol regulatory element-binding protein 2; Hmgcoar (Hmgcr) - 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase); Cyp7a1 - Cholesterol 7 alpha-hydroxylase/Cytochrome P450 Family 7 Subfamily A Member 1 (Cyp7a1); pre-Cyp7a1 – Cyp7a1 precursor mRNA; Fxrα1-2 and Fxrα3-4 - farnesoid X receptor (isoforms 1-2 and 3-4). *p<0.05.*

The relative mRNA levels of Srebp-2 were reduced with the western and protein diet by 34% and 49% respectively, while showing increase with the ketogenic diet by 73% and high fat diet by 17%. Hmgcr mRNA level was decreased with protein dilution by 78%, with western diet by 72%, and with high fat diet by 26%. Cyp7a1 mRNA levels decreased with the protein free and ketogenic diet by 51%, with high fat diet by 69% and with the western diet by 46%. Cyp7a1 pre-mRNA levels were overall similar to their corresponding mRNA level. Different Fxr isoforms (Fxrα1-4) showed no notable difference in mRNA levels with the diets used.

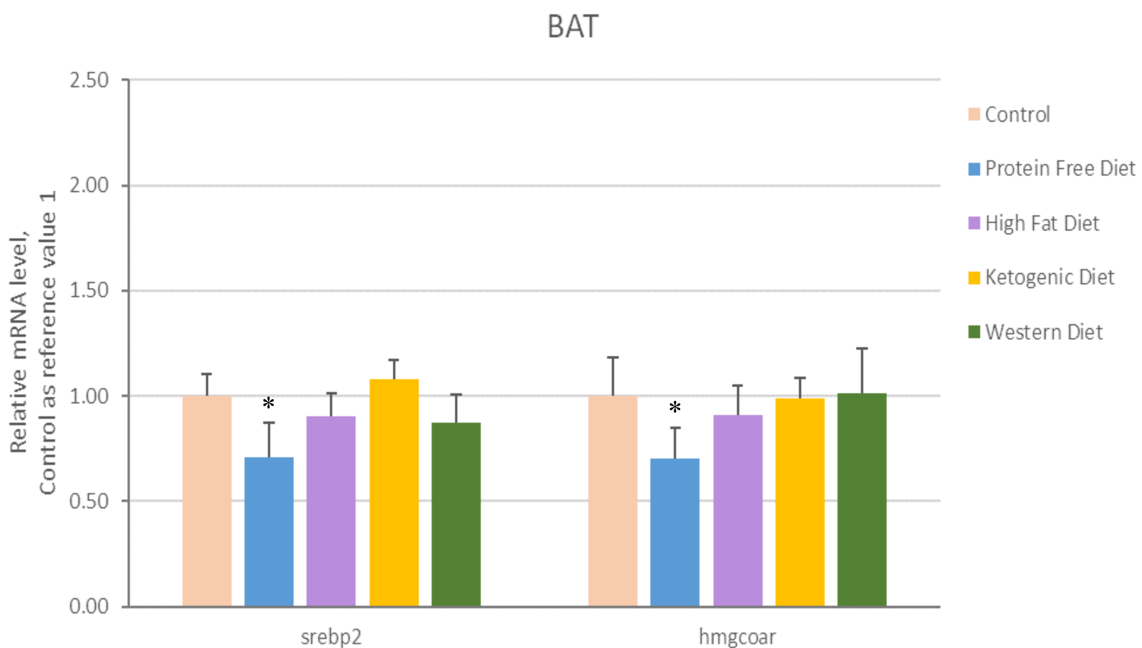

Figure 15: Overview of relative mRNA level of genes in cholesterol and bile acid metabolism in brown adipose tissue after feeding a protein free, high fat and ketogenic diet for 4 weeks and western diet for 5 weeks.

*Srebp2 - sterol regulatory element-binding protein 2; Hmgcoar (Hmgcr) - 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase). *p<0.05.*

For comparison, Srebp-2 and Hmgcr mRNA levels were investigated in BAT (as there is no bile acid synthesis in adipocytes). The mRNA levels of these genes were similar one to another, only showing a notable decrease with protein dilution. Srebp-2 mRNA level decreased with the protein free diet by 29%, while Hmgcr mRNA level decreased by 30%. In contrast to the liver, the western diet did not have a noticeable impact on mRNA levels of these genes.

3.4 Secreted Hormones & Proteins – Inter-organ Communication

This section targets three more investigated genes, which cannot easily be divided into the aforementioned categories. Fibroblast growth factor 21 (FGF21) is a hepatokine that, amongst other metabolic functions, increases glucose uptake exclusively in the adipose tissue. In the liver, FGF21 induces fatty acid oxidation, while inhibiting lipogenesis. Insulin-like growth factor 1 (IGF-1) is another hepatokine, that has a similar structure to insulin and stimulates cell proliferation and has anabolic effects (e.g., stimulates lipogenesis). IGFBP1 (insulin-like growth factor binding protein 1) binds insulin-like growth factors in order to prolong their half-life and alter their affinities to receptors.

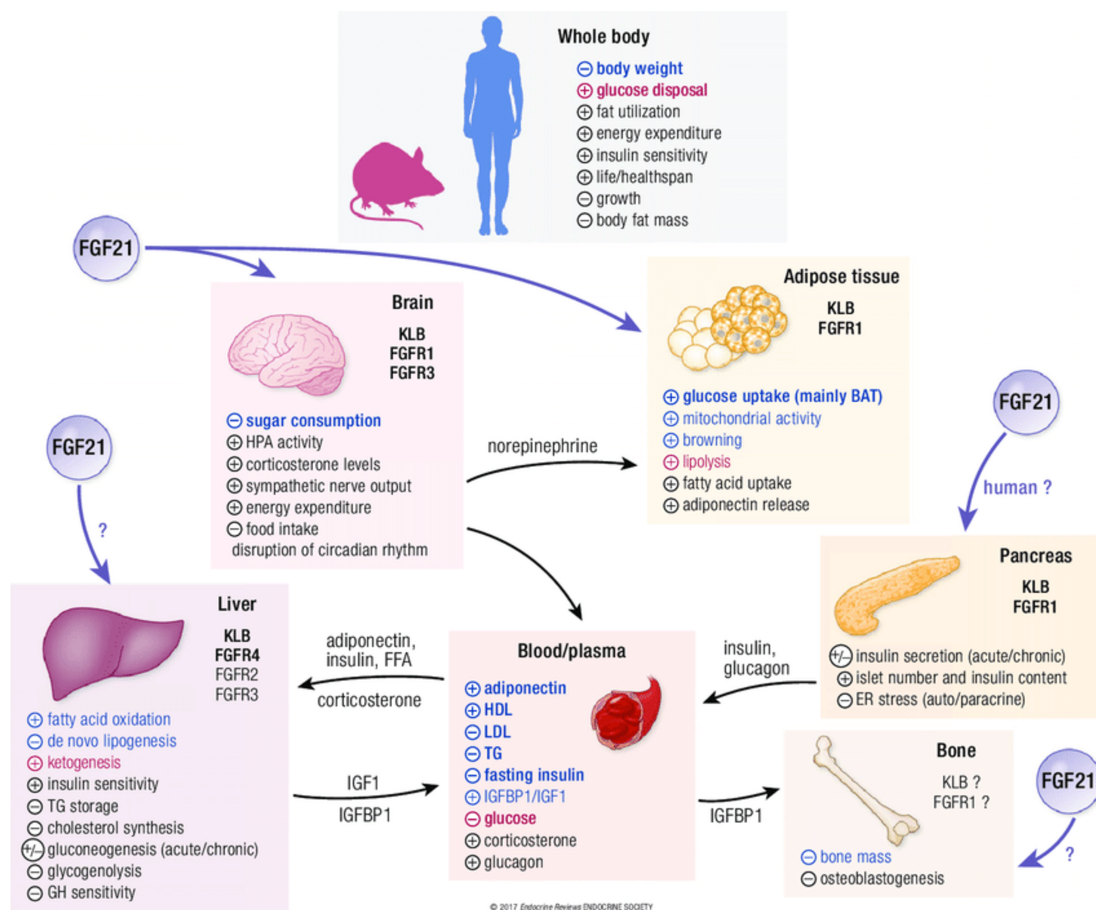

Figure 16: Various effects of FGF21 and its connection to IGF-1 and IGFBP1.
The increase in mitochondrial activity in BAT could prove useful, given the positive effects of BAT activation on lipoprotein flux and serum cholesterol levels (adapted from Staiger H.) [47].

As seen in the figure, FGF21 has many metabolic effects and interactions, which are still being investigated. We have conducted research of this gene to

complement the existing evidence and add more insight on the effects of other special diets on the mRNA expression.

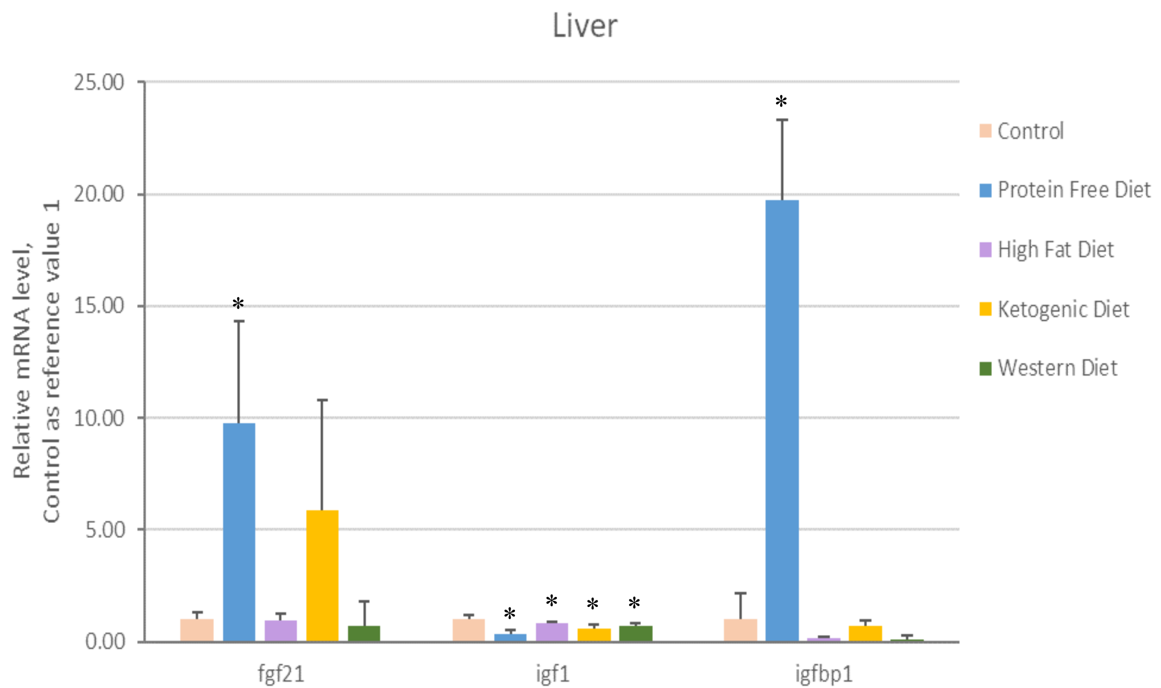

*Figure 17: Overview of relative mRNA level of Fgf21 (fibroblast growth factor 21), Igf1 (insulin-like growth factor) and Igfbp1 (insulin-like growth factor-binding protein 1) in the liver. *p<0.05.*

Relative Fgf21 mRNA levels increased massively with the protein free diet, showing more than nine times the mRNA (+877%) compared to control. The ketogenic diet increased Fgf21 mRNA levels by 486%, while a western diet decreased mRNA levels by 27%. The relative Igf1 mRNA level decreased by 66% with the protein free diet, while the ketogenic and western diet lead to a 44% and 30% decrease respectively. Protein dilution increased Igfbp1 mRNA levels dramatically showing 19.73 times higher values (+1873%) compared to control. The changes in Igf1 and Igfbp1 mRNA levels with the protein free diet show an inversely proportional relationship. The high fat, western and ketogenic diet decreased relative Igfbp1 mRNA levels by 83%, 89% and 32% respectively. Fgf21 showed no expression in brown adipose tissue.

4 Discussion

The relevant results of this diploma thesis will be discussed with emphasis on the isoforms mostly affected by dietary regimes and their differences in regulation between tissues investigated in this diploma thesis.

4.1 Chrebp-α and Chrebp-β

As mentioned before, the mRNA levels of Chrebp-α and β were strongly influenced by the diets used. The transcription factors carbohydrate response element binding protein α and β are glucose activated and are involved in hepatic lipogenesis and thermogenesis in BAT [45, 48].

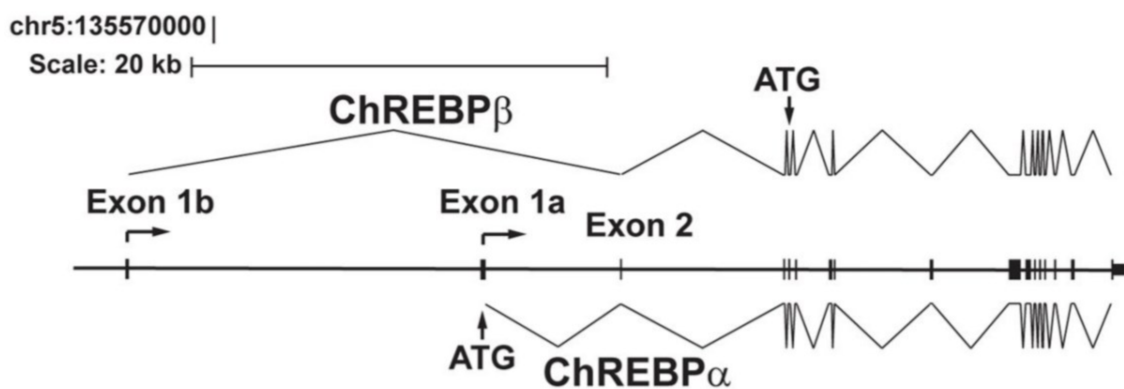

Figure 18: Human Chrebp alternative promoter mediated transcription leads to two isoforms with unique properties (adapted from Herman M. with modifications) [49].

Because Chrebp transcription in mice follows similar mechanisms to those in humans, mRNA levels of both isoforms in liver and BAT were analyzed and compared to explore if there is a dietary effect on promoter usage. It was believed that glucose activated Chrebp-α induces Chrebp-β expression [49]. More recent studies show, that Chrebp-β also has regulatory mechanisms independent of Chrebp-α, or even have reciprocal regulation between each other in some tissues instead [50]. The results of this section aim to investigate this regulation and the relationship of these isoforms in liver and brown adipose tissue on a dietary level.

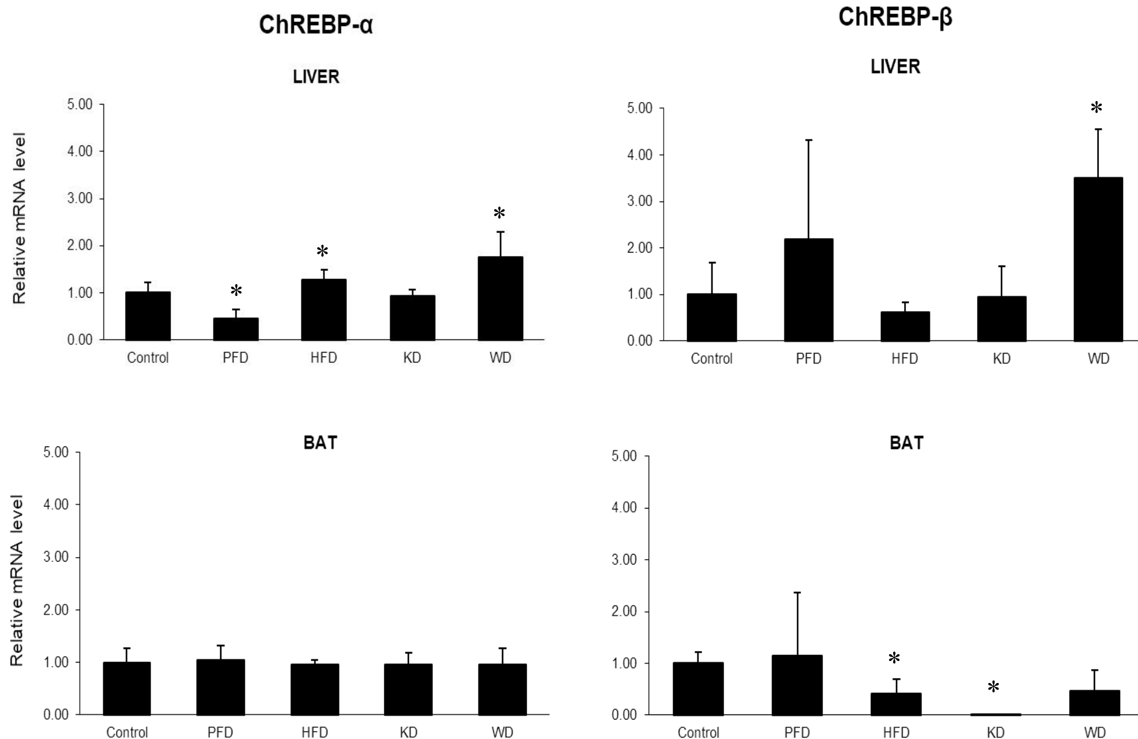

*Figure 19: Relative Chrebp-α and β mRNA expression in liver and BAT in side-by-side comparison. The western diet showed an increase in mRNA levels in both isoforms in the liver. An inversely proportional effect can be seen when comparing the mRNA levels of both isoforms after feeding a protein free diet in the liver (ratio 1:4.84). Furthermore, when comparing Chrebp-β mRNA levels in the liver and BAT, an inversely proportional relationship can be seen with the western diet (ratio 1:7.46), while the ketogenic diet suppressed mRNA levels only in BAT. Chrebp – carbohydrate-response element-binding protein. *p<0.05.*

Comparing mRNA levels of Chrebp-α and Chrebp-β in the liver relative to their corresponding controls, indicates that the protein free diet and high fat diet could have inversely proportional effects on alternative promoter activation in the liver. It is worth noting, that the PCR results of Chrebp-β showed low mRNA levels in the liver with high Cp-values at 30.16 for control, 30.00 for HFD and 29.27 for KD. The PFD reduced, while the WD and in lesser extent the HFD increased mRNA levels of Chrebp-α and Gk in the liver in a similar matter. This similarity between Chrebp-α and Gk mRNA levels is explained by the shared glycolytic properties of these genes [51]. Pfkb1, which regulates glucose levels, also showed a similar trend in hepatic mRNA expression compared to Chrebp-α and Gk. Although it would be expected that Pck1, which is an important enzyme in gluconeogenesis, mRNA expression increases, the results did not show a notable change in mRNA levels in the liver. In BAT on the other hand, Pck1 levels increased with PFD and, to a

lesser extent, with HFD and WD, which in combination with low Cp-values means that BAT could have high gluconeogenic activity that increases with protein dilution. Furthermore, the western diet induced mRNA levels of both Chrebp isoforms in the liver which indicates a response to cholesterol and, possibly, a more complex mechanism for maintaining mRNA levels of these isoforms. The PFD raised relative Chrebp-β mRNA levels in the liver. A study has shown that a high fat diet and cyclic AMP can suppress Chrebp [52]. The results in BAT show that Chrebp-β mRNA levels seem to decrease proportional to dietary fat intake. In comparison to this, a study has shown that a high fat diet also decreases Chrebp mRNA expression in white adipose tissue [53]. The ketogenic diet, which contains the highest amount of fat (with a very low amount of protein and almost no glucose), completely suppressed Chrebp-β mRNA in BAT. While high Chrebp expression in the liver is associated with hepatic lipogenesis and metabolic syndrome, a study has shown a positive correlation between low ChREBP-β expression in adipose tissue and insulin resistance and type 2 diabetes in humans [54]. Thus, the results verify, that a diet high in fat leads to reduced Chrebp-β mRNA levels in BAT and can therefore be associated with metabolic diseases like diabetes. It is important to note, that the results in this diploma thesis are from BAT and not WAT, which is where most research has been made, because of its availability. The amount of sugar in the diet does not seem to influence Chrebp-β mRNA expression in BAT. In contrast, Chrebp-α mRNA levels in BAT have not been influenced by the diets used, suggesting independent regulation of the two Chrebp isoforms in BAT (e.g., Chrebp-β is activated by mTORC2) [55]. It has been shown that BAT has gluconeogenic properties, but the high mRNA expression of G6pdh shows that it is also active in the pentose phosphate pathway. The results have shown dramatic decrease in G6pdh mRNA expression up to 50% with KD, HFD and lesser decrease with PFD and WD. This decrease is comparable to the effects of adrenalectomy or thyroidectomy on G6PDH enzyme activity in adipose tissue [56]. Because Chrebp expression is involved in the development of metabolic syndrome, modulating its expression by dietary restriction, or furthermore specific nutrient restriction could prove useful in treating hepatic steatosis, insulin resistance or even cancer [45, 50, 57]. For this, further investigation of the tissue-specific regulation and interaction of these isoforms is needed and will be of interest in the future.

4.2 Hexokinase 2

It has been shown that reduction in Hk2 expression can help increase the efficacy of chemotherapy in cancer types, where Hk2 is overexpressed [58-60]. This effect is most probably due to anti-apoptotic properties, chemoresistance and involvement in the Warbung effect of Hk2 [61, 62]. The results of this diploma thesis show, that the ketogenic diet reduced Hk2 mRNA expression in BAT by more than 50%.

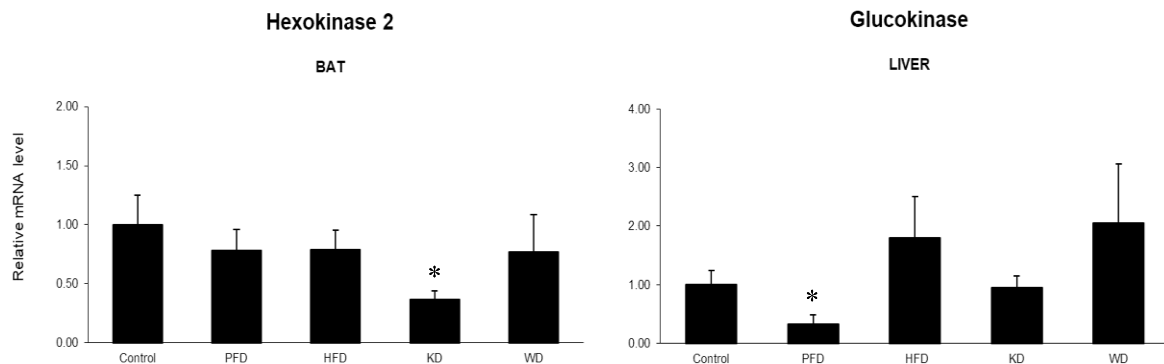

Figure 20: Relative Hk2 mRNA expression in BAT and comparison to Gk in liver.
*The ketogenic diet more than halved the expression of Hk2 in BAT. The protein free diet had a similar effect on Gk in the liver. Hk2 – hexokinase 2; Gk – glucokinase. *$p<0.05$.*

In comparison to Hk2, Gk mRNA level in the liver was not affected by the ketogenic diet. Other diets have influenced the mRNA levels of Gk, which do not seem to be tied to the dietary effects on Hk2. This could be explained by the additional properties which are exclusive to Hexokinase 2. This acknowledgement leads to the conclusion, that a ketogenic diet can be used in combination with selected chemotherapy in treating cancer. Because Hk2 is also expressed in the skeletal muscle and heart, the effects of ketogenic diet should be investigated in these tissues as well, so this therapy concept can be developed further.

4.3 Srebp-1 and Srebp-2

SREBPs (sterol regulatory element-binding proteins) are important transcription factors involved in the lipid and cholesterol metabolism. This family of transcription factors includes two genes, Srebp-1 and Srebp-2. The former has two important isoforms called Srebp-1a and Srebp-1c. It has been shown, that SREBP-1a and -1c influence fatty acid synthesis and lipogenesis, while SREBP-2, but also SREBP-1a control the synthesis of cholesterol [63]. Furthermore, tumor cells have altered glucose and lipid metabolism involving Srebp overexpression. Suppressing Srebp showed positive effects on inhibiting tumor growth by inducing cell death [64]. In this diploma thesis, dietary effects on the mRNA levels of both Srebp-1 isoforms and Srebp-2 in the liver and BAT were investigated and compared.

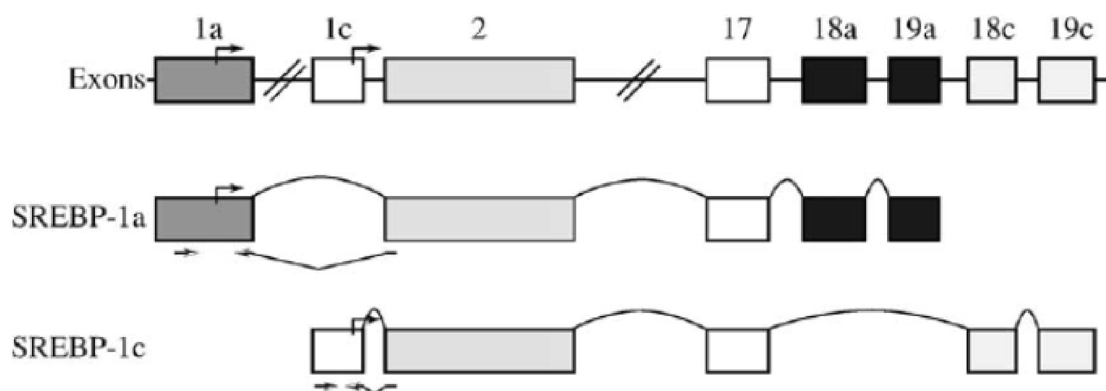

Figure 21: An overview of the two major Srebp-1 isoforms as a product of alternative splicing (adapted from Felder T. with modifications) [65].

Considering the previous investigation of these isoforms, including the different expressions in various tissues and some dietary effects, we conducted research of the mRNA levels of both isoforms in the liver and BAT.

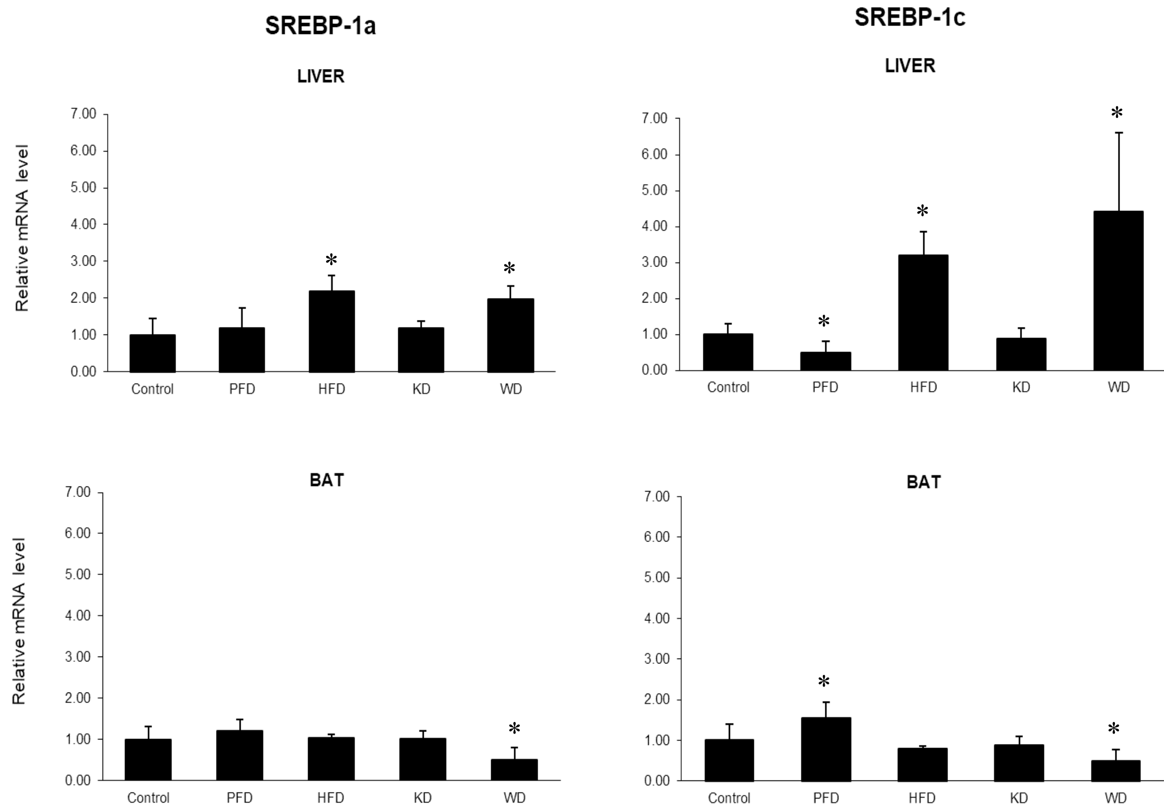

Figure 22: Relative Srebp-1a and Srebp-1c mRNA expression in liver and BAT in side-by-side comparison.

*The western and high fat diet increased mRNA levels of both isoforms in the liver. When comparing the effects of the western diet on mRNA levels in the liver and BAT, an inversely proportional effect can be seen in both isoforms (ratio in Srebp-1a 1:4.00; ratio in Srebp-1c 1:9.04). The protein free diet also showed an inversely proportional effect on the mRNA levels of Srebp-1c in these tissues (ratio 1:3.16). Srebp – sterol regulatory element-binding protein. *p<0.05.*

The mRNA levels of Srebp-1c in the liver showed a dramatic increase when fed a western diet rich in cholesterol. The mRNA levels of Srebp-1a also increased, but to a much lesser extent. Interestingly, the mRNA levels of the genes involved in lipogenesis (e.g., Scd1, Fasn and Acly) were not notably influenced by the western diet. The ketogenic diet suppressed mRNA levels of all three genes in BAT, while Fasn and Acly were not notably influenced in the liver.

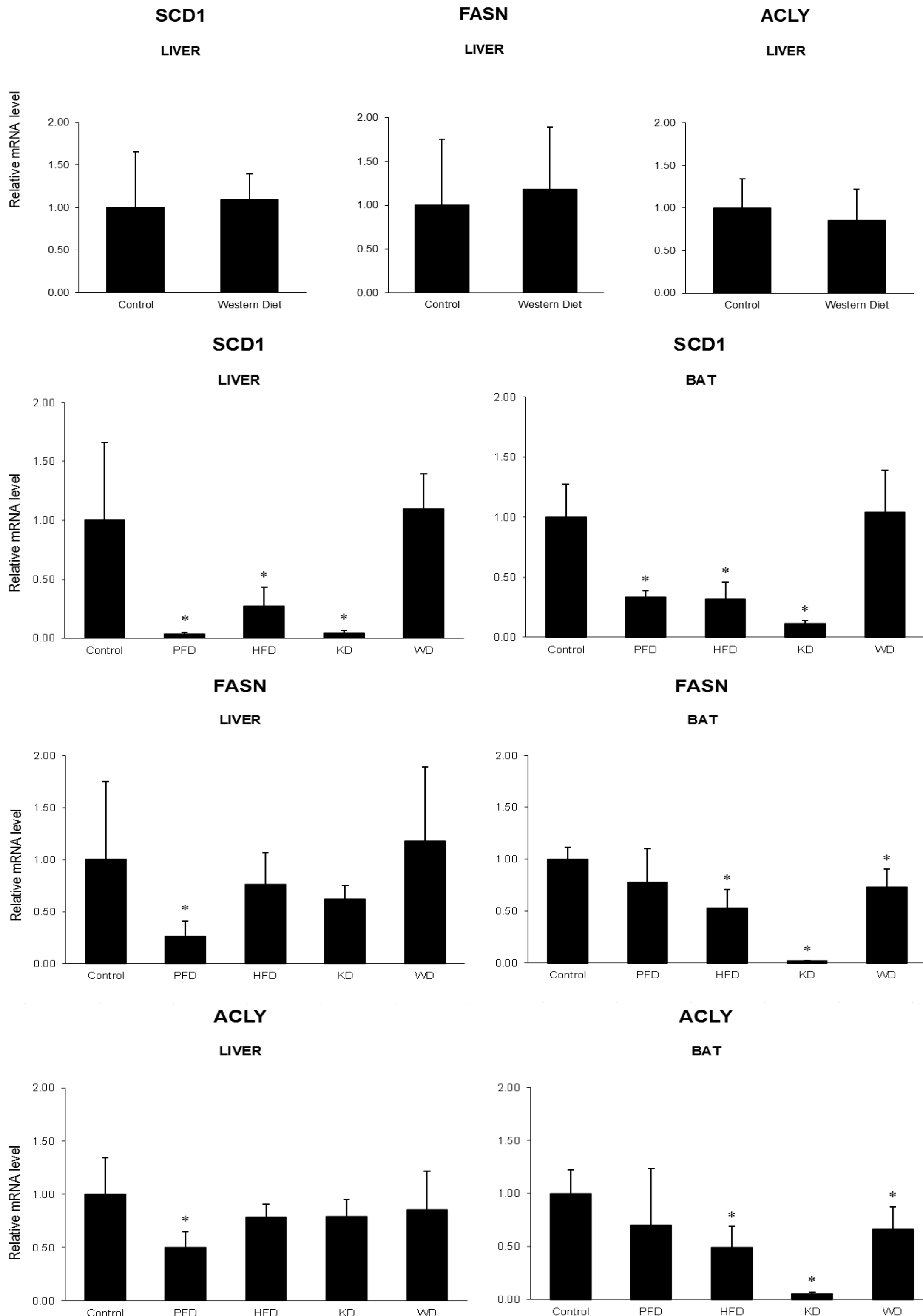

Figure 23: Relative mRNA level of Srebp-1 regulated genes Scd1, Fasn and Acly.

*The mRNA levels of these genes were not influenced by the western diet (1.25% cholesterol) in the liver. All other diets have decreased mRNA levels of these genes in the liver (Scd1 was affected the most). The ketogenic, high fat and western diet suppressed these genes in BAT, which shows their response to dietary fat. The protein free diet also suppressed Scd1 in BAT. Scd1 - stearoyl-CoA desaturase-1; Fasn - fatty acid synthase; Acly – ATP citrate lyase. *p<0.05.*

These results show that the mRNA levels of these genes are not directly proportional to those of Srebp-1 under a cholesterol enriched diet. This indicates that Srebp-1 mRNA levels or even mRNA processing could change with the amount of dietary cholesterol, which does not affect mRNA levels of other lipogenic genes. These findings are interesting, since it is known that Srebp-2 regulates cholesterol synthesis instead. Srebp-2 mRNA levels in the liver halved with the western diet, showing an inversely proportional effect compared to Srebp-1 isoforms. More typical was the increase in Srebp-1 mRNA levels after feeding a high fat diet. Both isoforms showed increased mRNA levels in the liver, but not in BAT. This is explained by SREBP-1 being responsible for lipogenesis in the liver with dietary fatty acid overflow. Why Srebp-1 mRNA levels were elevated after feeding a cholesterol enriched diet remains unclear and needs to be investigated further. Some mechanisms involved could be decreased Srebp-1 mRNA half-life to preserve mRNA levels of this transcription factor in order to have a ready amount of mRNA for translation, when cholesterol intake is high. When comparing Srebp-1c mRNA levels in the liver and BAT, a roughly inversely proportional relationship can be noticed across all diets used, except for the ketogenic diet. This relationship does not seem to be linear, as the differences in the liver seem to be much more drastic, compared to those in BAT. The inversely proportional relationship might as well be a coincidence, considering that both Srebp-1a and Srebp-1c mRNA levels in BAT show similar results overall and could be independent of the results in the liver.

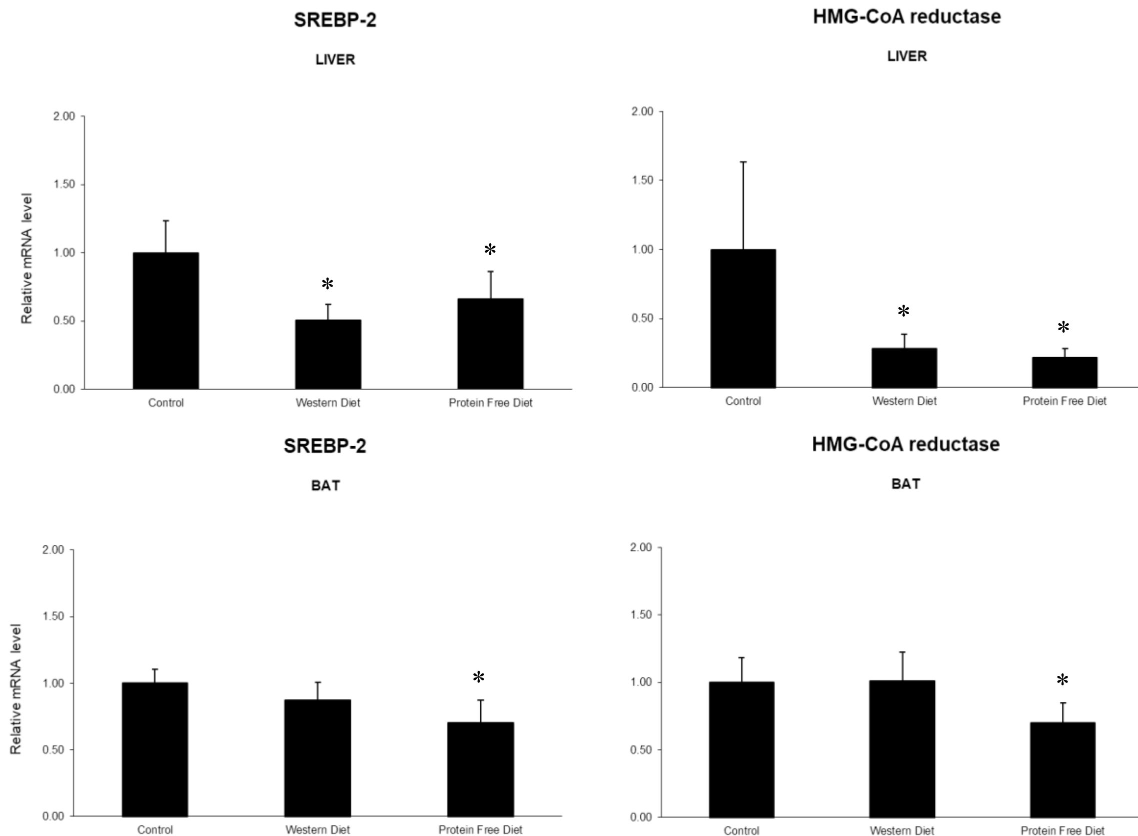

Figure 24: Srebp-2 and Hmgcr mRNA levels in the liver and BAT.
*The mRNA levels of both Srebp-2 and Hmgcr decreased in the liver by feeding a western diet rich in cholesterol (1.25%) and protein free diet for 5 and 4 weeks respectively. In comparison, mRNA levels of these genes in BAT did not decrease as much. Srebp – sterol regulatory element-binding protein; HMG-CoA reductase (Hmgcr) - 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase. *p<0.05.*

The mRNA levels of both Srebp-2 and Hmgcr, which are involved in cholesterol synthesis decreased, showing the inverse relationship between dietary cholesterol intake and cholesterol synthesis. Interestingly, the results show a similar decrease with protein dilution. In BAT, only the western diet decreased mRNA levels of Srebp-2 and Hmgcr, but to a much lesser extent compared to the liver. These findings are interesting, since it has been shown that Srebp-2 is upregulated in prostate cancer cells with a loss of negative feedback by cholesterol [66]. Furthermore, the inhibition of Srebp-2 through Fatostatin slows down prostate cancer growth and invasion [67]. Preclinical research suggests that statins, which inhibit HMG-CoA reductase, also show positive effects in combination with chemotherapy in some cancer types [68, 69]. The effects of the protein free and

western diets could prove useful for further investigation and complement the involved therapies.

4.4 Acly and its Isoform

As mentioned before, custom primers have been designed, to separately determine mRNA levels of ATP citrate lyase, which connects the carbohydrate and lipid metabolism. It catalyzes the reaction of CoA and citrate, producing oxaloacetate and acetyl-CoA. The latter is an important molecule in *de novo* fatty acid synthesis and cholesterol synthesis.

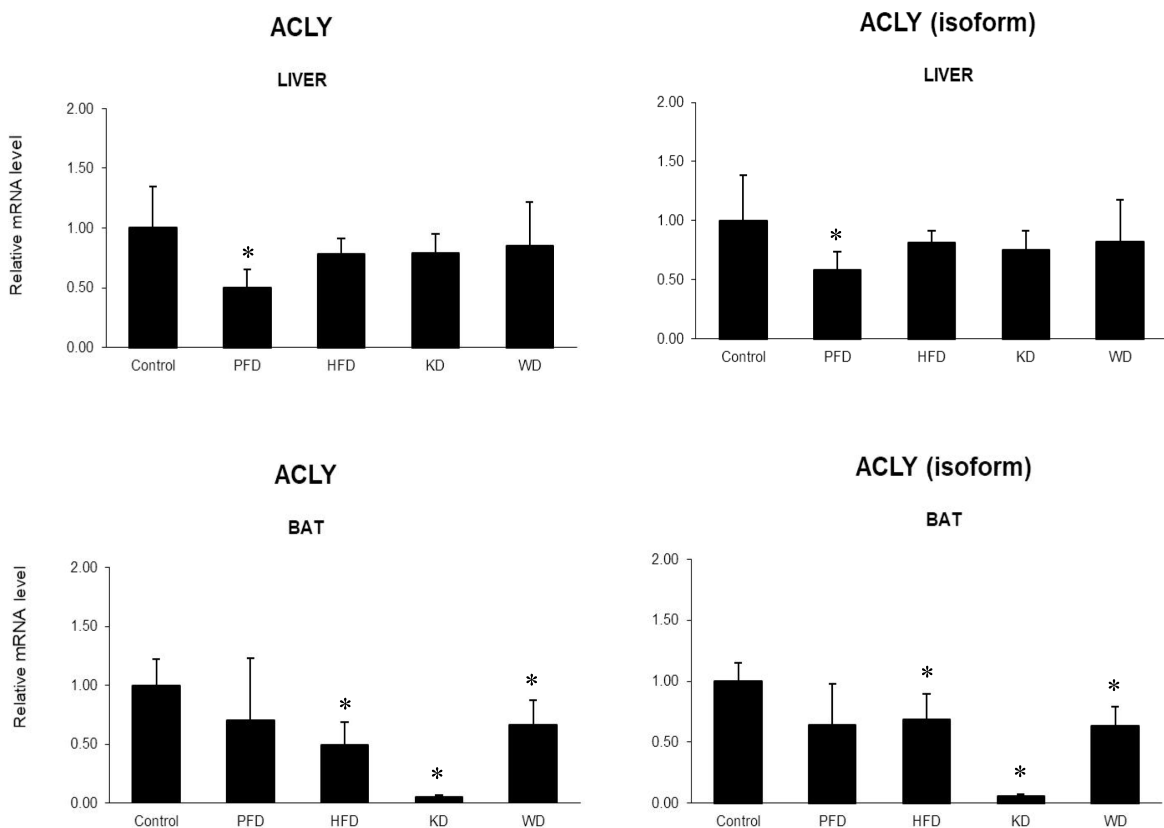

Figure 25: The differences between mRNA levels of the two Acly isoforms were investigated in both tissues to highlight potential differences in the regulation of these isoforms provoked by special diets.
*There were no notable differences in relative mRNA levels of Acly and its isoform. In general, a response to dietary fat can be seen in BAT, while the protein free diet seemed to play a bigger role in suppressing this gene in the liver. Acly – ATP citrate lyase. *p<0.05.*

The results imply that there is no difference in mRNA expression of the two Acly isoforms. Another explanation is that the primers used were not isoform-specific,

leading to nearly identical qPCR results. Nonetheless, the protein free diet decreased mRNA levels in both investigated tissues. The amount of dietary fat decreased mRNA levels of Acly in BAT showing the negative feedback regulation of lipogenesis through dietary fat intake.

4.5 Cyp7a1 and Fxr

Cholesterol 7 alpha-hydroxylase (CYP7A1) is a cytochrome P450 enzyme which is essential in bile acid synthesis from cholesterol. This oxidoreductase catalyzes the reaction of cholesterol to 7-alpha-hydroxycholesterol, which is the most important step in the synthesis of bile acids. Bile acids can also inhibit Cyp7a1 transcription through a feedback loop mechanism, involving farnesoid X receptor (Fxr) and small heterodimer partner (SHP) [70, 71].

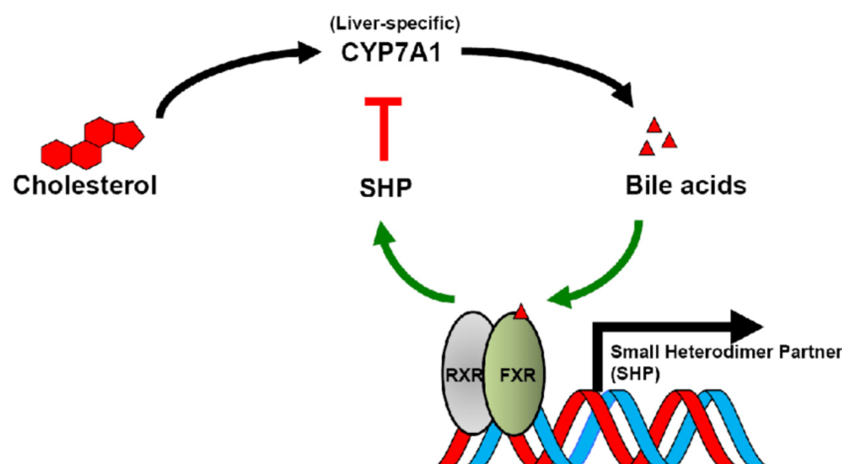

Figure 26: Cyp7a1 negative feedback loop regulation.
CYP7A1 is the rate-limiting step of the classical pathway of bile acid synthesis. The synthesized bile acids activate Fxr transcription, which increases the expression of small heterodimer partner (SHP). These reduce the expression of Cyp7a1, which consequently reduces bile acid synthesis [71].

There are also other regulatory mechanisms of this gene, which involve fibroblast growth factor 15 and 19 (FGF15, FGF19), protein kinase C (PKC) and c-Jun N-terminal kinase (JNK) signaling pathways and epigenetic regulation [70]. Research has shown an induction of Cyp7a1 mRNA in rats through short termed dietary cholesterol, which indicates a purely transcriptional regulation. In contrast, a long-term cholesterol-rich diet suppresses Cyp7a1 mRNA levels [72]. Interestingly, the way the mRNA sequences are built show signs of short-life mRNAs [70]. In this

research, Cyp7a1 mRNA and pre-mRNA levels were determined in order to investigate the transcriptional regulation and possible mRNA modification mechanisms of this gene.

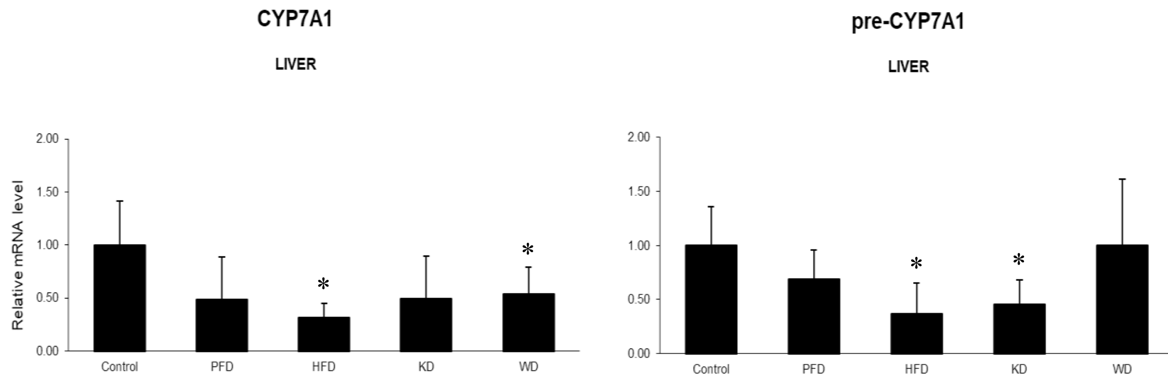

Figure 27: Relative Cyp7a1 mRNA and pre-mRNA levels in the liver.
*The mRNA and precursor mRNA levels of Cyp7a1 were measured in the liver after feeding protein free, high fat, ketogenic diets for 4 and a western diet (1.25% cholesterol) for 5 weeks. All diets suppressed Cyp7a1 mRNA and pre-mRNA levels, where the high fat, ketogenic and western diet had the biggest impact. *p<0.05.*

The results show a decrease in Cyp7a1 mRNA levels across all diets used. Interestingly, the western diet did not suppress Cyp7a1 pre-mRNA levels, while the other diets show a relatively similar trend, compared to mRNA levels. This means, that the turnover of pre-mRNA to mature mRNA could have been slowed down by chronic dietary cholesterol intake. This could be another regulatory mechanism of Cyp7a1 at the early post-transcriptional level. It would be interesting to study intestine (e.g., Fgf15) in follow-up.

Another point is that the alternative pathway in bile acid synthesis, involving sterol 27-hydroxylase (CYP27A1), could be influenced by these diets. Other genes involved in bile acid synthesis, including Cyp27a1, Fgf15/19, Pkc and JNK, should be investigated to better understand the effects of dietary regimes on their regulations.

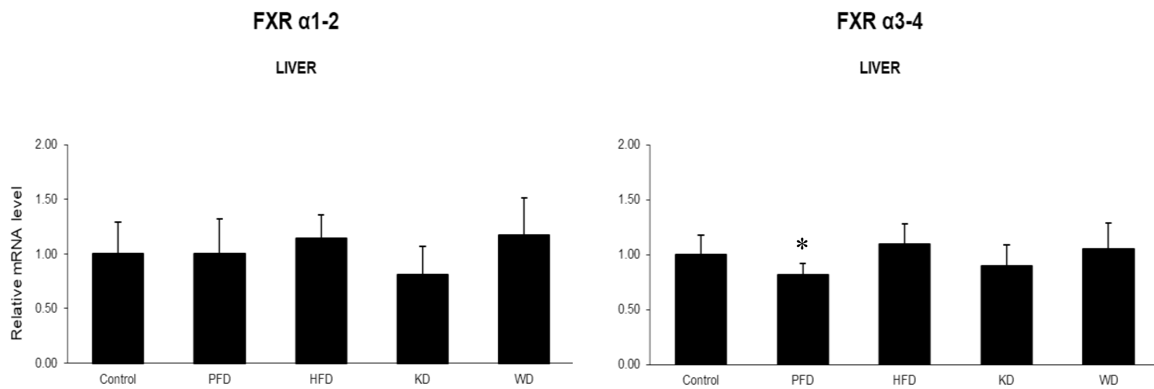

Figure 28: Fxr isoform mRNA levels in the liver.
*The relative mRNA levels of the Fxr isoforms did not notably change with the diets used. *p<0.05.*

The lack of increase of Fxr mRNA levels indicates that the negative feedback through Fxr is not a plausible explanation for the decrease in Cyp7a1 mRNA levels, at least regarding mRNA levels.

4.6 Fgf21

Fibroblast growth factor 21 is a member of the FGF superfamily of peptides and a hepatic hormone involved in fatty acid oxidation, gluconeogenesis, ketogenesis and also regulates insulin mediated glucose uptake in adipocytes [73, 74]. It is also known that FGF21 leads to decrease in appetite for sugar and sweeteners [75]. Previous studies have shown an increase in Fgf21 expression when feeding a ketogenic diet, but also with protein dilution [73, 76]. Higher expression of Fgf21 is associated with weight loss and is an important factor in lipid homeostasis. The findings of this diploma thesis also show massively elevated Fgf21 mRNA levels in the liver when feeding a protein free and ketogenic diet, hardening the results of the previous studies.

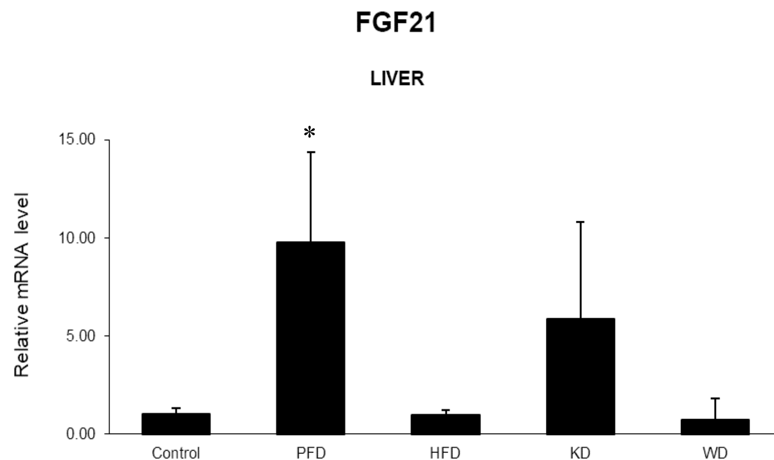

Figure 29: Fgf21 mRNA levels in the liver.
*The mRNA levels of Fgf21 massively increased in the liver with the protein free and ketogenic diet. The western diet seemed to insignificantly decrease mRNA expression of this gene in the liver. Fgf21 – fibroblast growth factor 21. *p<0.05.*

These studies have shown that a diet that simulates fasting (e.g., ketogenic or protein free diet) can increase FGF21 serum levels in mice through fatty acids and peroxisome proliferator-activated receptor α (PPARα) [47, 77]. The induction of FGF21 in humans was only observed with the protein free, but not ketogenic diet [47]. FGF21 inhibited growth and lead to increased longevity in mice, by influencing the T lymphocytes [47]. Furthermore, FGF21 inhibited lipogenesis in the liver, through Fasn and Srebp-1c suppression [47]. The result of this diploma thesis shows similar effects, where the protein free and ketogenic diets increased Fgf21 but decreased Srebp-1c and Fasn mRNA levels in the liver. Interestingly, Srebp-1c mRNA levels increased in BAT with the protein free diet. It would have been interesting to see Fgf21 expression in BAT using these diets, because of its role in glucose uptake in this tissue. While FGF21 has shown positive effects on lipid levels, there is evidence that this growth hormone does not decrease glucose levels in the blood, or even be a factor in the development of Type 2 diabetes mellitus [47, 78]. Other effects of FGF21, like decreasing bone density, association with pancreatitis, lipodystrophy, muscle, and mitochondrial diseases have also been described [47]. In conclusion, the effects of FGF21 in mice and humans needs to be investigated further, in order to make it a viable target in the therapy of hepatic steatosis or other metabolic diseases.

4.7 Igf1 and Igfbp1

Insulin-like growth factors are, as the name implies, proteins with a similar sequence to insulin. IGF-1 has multiple functions in promoting cell proliferation and inhibiting apoptosis and is produced in the liver under regulation of somatotropin and other hormones [79]. Dietary behavior also changes Igf1 expression, which made it an interesting target in this research. IGF-1 binds to the IGF-1 receptor, but also to other receptors, although with a lower affinity. IGF binding proteins are regulatory proteins, which influence the function of IGFs. It has been shown that dietary restriction or special diets lead to generally lower serum IGF-1 and higher insulin dependent IGFBP1 levels, which can prove relevant in chemotherapy [80, 81]. The results of this thesis show similar results, where the protein free diet decreased Igf1 and increased Igfbp1 mRNA levels.

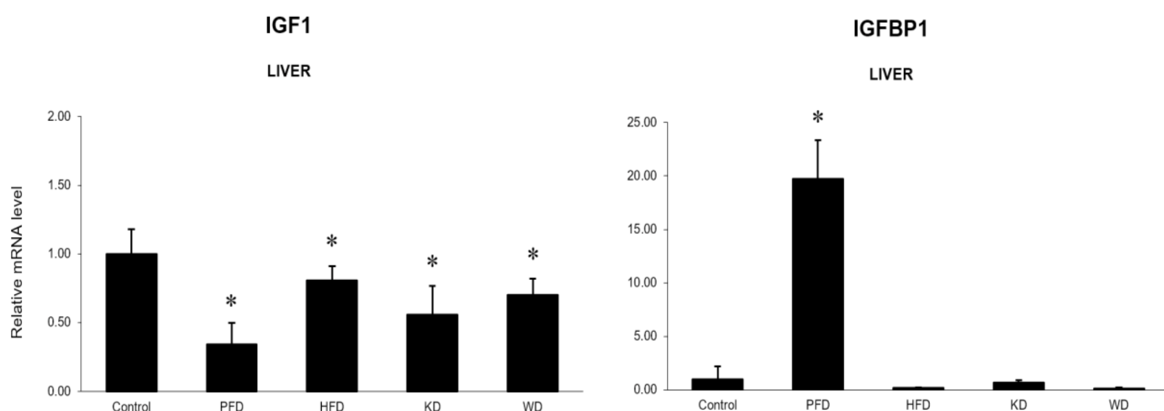

Figure 30: Igf1 and Igfbp1 mRNA levels in the liver.
*The protein free diet decreases Igf1 and increases Igfbp1 mRNA levels in the liver in an inversely proportional manner (ratio 1:58). Overall, the mRNA levels between these genes seemingly stand in an inversely proportional relationship with all diets used. *p<0.05.*

4.8 Differences and Similarities between Diets and Tissues

The effects of special diets in two tissues, namely the liver and BAT, have been investigated in order to see their potential interactions. There are many studies that have been aiming at showing these effects in the liver, but not so many for the brown adipose tissue. The results of this diploma thesis make it clear that not only the liver, but also BAT plays an important role in the metabolic regulation of many genes, as dietary regimes showed significant changes in gene expression in both tissues. Some genes have been influenced in interesting ways, showing inversely

proportional relationships between the two tissues with special diets. For example, the cholesterol-enriched western diet increased the expression of Chrebp-β in the liver but decreased in BAT with a ratio of 1:7.46. The expression of Chrebp-α showed an inversely proportional relationship compared to Chrebp-β with the protein free diet in the liver with a ratio of 1:4.84. The response of Chrebp to glucose in carbohydrate-rich diets was mainly seen in the liver, where the western diet increased the expression of Chrebp (β>α, with a ratio of 1:2.00). In BAT, the expression of Chrebp-α was not changed at all with these diets, while the ketogenic, high fat and western diet suppressed Chrebp-β, seemingly linked to the amount of dietary fats.

The inversely proportional relationship between these tissues could also been observed in the expression of Srebp-1 isoforms with a western diet. Srebp-1a expression increased with the western diet in the liver, while getting suppressed in BAT with a ratio of 1:4.00. Similarly, the expression of Srebp-1c with the western diet increased in the liver and decreased in BAT with an even higher ratio of 1:9.04. Contrary, the protein free diet suppressed Srebp-1c in the liver while inducing its expression in BAT with a ratio of 1:3.16. The western diet, which is rich in sugar, increased the expression of Srebp-1 and both Chrebp isoforms in the liver, promoting lipogenesis. Contrary, the expression of these genes (excluding Chrebp-α expression, which remains the same) tends to decrease with the western diet in BAT.

These findings imply that a potential communication between these tissues exists at the genetic (transcriptional) level. The genes involved in cholesterol biosynthesis, Srebp-2 and Hmgcr did not show this kind of linkage in expression between the tissues. The expression of Acly seemed to decrease with the amount of dietary fats in BAT, but not in the liver.

Interestingly, the protein free diet had the most dramatic impact on Igf1 and Igfbp1 expression. This is most likely due to the decrease in growth caused by protein deficiency through the suppression of Igf1 and somatotropin, while Igfbp1 tends to be expressed inversely proportional. A similar, yet less dramatic, effect could be seen with the ketogenic diet, which also mimics fasting.

The expression of Fgf21 increased with the protein free and ketogenic diet in the liver. FGF21 has shown to activate BAT by increasing mitochondrial activity in adipose tissue, which leads to benefits regarding plasma cholesterol and TAG

levels. Thus, it would be interesting to investigate the dietary effects of this gene in BAT and WAT. Fgf21 expression increased with the ketogenic diet in the liver and simultaneously decreased Chrebp-β and DNL gene expression in BAT, but not in the liver. It is known that FGF21 increases glucose uptake and browning in BAT as well as lipolysis in mice [47]. The suppression of Chrebp-β and genes involved in DNL in BAT and increased Fgf21 expression in liver could be correlated, complementing the lipolytic effects of FGF21 in BAT and liver. This effect is specific to BAT, as Chrebp expression in the liver was more sensitive to the amount of dietary sugar, than fats. Furthermore, it is known that Fgf21 is regulated in the liver by glucose and ChREBP in the presence of PPARα [82]. As the results show, the expression of the Chrebp isoforms was different between the tissues, possibly indicating a special regulation of Fgf21 by these isoforms in BAT. As BAT can be activated pharmacologically and through cold exposure, it would be interesting to investigate the response of these interactions to changes in BAT activity.

4.9 Limitations

The limitations of this diploma thesis are tightly bound to the methods and animals used. Quantitative PCR was used to determine mRNA levels of the investigated genes, which gives insight at the transcriptional level, but does not give any information at the protein level. This means, that protein western blotting of the relevant genes would be useful for further investigation. Although the mice were fed the same diet and had the same habitat within a group, the individual differences of the mice were not considered. This has benefits regarding randomisation, but limitations regarding metabolic states or eventual pathologies of the mice used. For example, mice that had developed diseases like insulin resistance, metabolic syndrome, could have had different cell behaviours, which were not considered. It would have been interesting to see how other tissues (e.g., white adipose tissue, intestine) would have responded to these diets, as some genes show expression in these tissues. Moreover, it is important to notice that gene expression can differ between humans and mice. Because the animal number was fairly small, the discrepancies in the mRNA levels of the investigated genes could have shown effects, which would have not been there physiologically. Furthermore, the animals were sacrificed, and their tissues cryopreserved at a

specific point of time, which means that the mRNA levels were measured only once, giving no insight in the circadian rhythm, or other factors over time. Conclusively, this research is not based on mechanistic evidence, and the data it provides should be used for further research purposes.

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