The effects of different diets on obesity and liver steatosis in mice

A diet rich in sucrose, fat and cholesterol induces liver steatosis

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Master in clinical nutrition

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1 Abstract

Introduction: In Norway, 58 % of men and 47 % of women are classified as overweight or obese, which is a large increase since the mid-1980s. An often occurring consequence of obesity is accumulation of lipids in liver, which is often categorized as non-alcoholic fatty liver disease (NAFLD). NAFLD can further develop into NASH and cirrhosis, which is a leading cause for liver transplantations. The only current treatment against NAFLD is lifestyle change and a healthier diet. Investigating how different diets affect bodyweight and liver health is therefore important for public health.

Materials and methods: Liver tissue was sampled from a total of 89 female wild-type C57BL/6N mice from five independent diet-intervention studies, in which mice were either fasted, or fed a high fat-cholesterol-sucrose (HFS) diet, a high fat diet (HF), a high fat ketogenic diet (HFK) or a methionine and choline deficient diet (MCD). We measured bodyweight, fat mass, liver mass, liver TAG, and hepatic gene and protein expression. Expression of selected genes was measured with RT-qPCR, while expression of the lipid droplet binding proteins Plin2 and Plin3 was measured with western blotting.

Results: 24 hours of fasting led to reduced bodyweight and fat mass, and increased accumulation of fatty acids incorporated into triacylglycerol in liver. This was accompanied by increased expression of genes related to lipid droplet binding and lipid oxidation, and a decreased expression of genes related to lipogenesis. When comparing the diets with a high fat content, the HFS diet was the only diet that increased bodyweight and resulted in accumulation of liver fat. Furthermore, all high fat diets increased expression of genes related to lipid droplet binding and fatty acid oxidation. The HFK and HF diets with a low content of carbohydrates reduced the expression of the fatty acid synthesis genes *Acc* and *Fasn*, while this was not the case for the carbohydrate-rich HFS diet. The MCD diet, which is a model of NASH, induced a substantial loss of fat mass and increased hepatic fat accumulation, as well as increased inflammatory gene expression. Lastly, all diets increased the abundance of the Plin2 protein in liver, which indicate increased levels of lipid droplets.

Conclusion: A diet rich in fat, cholesterol and sucrose led to increased adiposity and more severe steatosis compared to high fat or ketogenic diets. Such a diet, often termed “Western

diet", also induced the expression of genes related to lipogenesis, which may indicate that high sucrose feeding favours active lipogenesis in liver regardless of the level of lipid intake. Only the MCD diet resulted in significant inflammation in parallel with accumulation of liver fat, indicative of NASH. In future studies, serum cholesterol, insulin resistance and liver histology should be measured to get a broader understanding of the metabolic effects these various diets have on the overall liver health.

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Martine Villemo Øksenvåg Ingebrigtsen

List of abbreviations

AAs: Amino acids

Abhd5: Abhydrolase Domain Containing 5

ACATs: Acetyl-coenzyme A acetyltransferases

Acc: Acetyl-CoA carboxylase

Acox1: Peroxisomal acyl-coenzyme A oxidase 1

Acetyl CoA: Acetyl coenzyme A

ANOVA: One-way analysis of variance

ATGL: Adipose triglyceride lipase

ATP: Adenosine triphosphate

BAT: Brown adipose tissue

BCA: Bicinchoninic acid

BCAA: Branched chained amino acids

BMI: Body mass index

BSA: Bovine serum albumin

Cidea: Cell Death Inducing DFFA Like Effector A

Cideb: Cell Death Inducing DFFA Like Effector B

Cidec: Cell Death Inducing DFFA Like Effector C

cDNA: Complementary deoxyribonucleic acid

Chrebp: Carbohydrate-responsive element-binding protein

CO_2: Carbon dioxide

Cpt1a: Carnitine palmitoyltransferase I

Dgat: Diglyceride acyltransferase

DIO: Diet-induced obesity

dNTP: deoxynucleoside triphosphate

DTT: Dithiothreitol

ER: Endoplasmic reticulum

EtOH: Ethanol

FA: Fatty acids

Fasn: Fatty acid synthase

$FADH_2$: Dihydroflavine-adenine dinucleotide

FFA: Free fatty acids

G0s2: G0/G1 Switch 2

GLUT2: Glucose transporter 2

GPD: Gross domestic product

HF: High fat

HFS: High fat-cholesterol-sucrose

HFCS: High fructose corn syrup

HFK: High fat ketogenic

HSL: Hormone-sensitive lipase

HUNT: The Trøndelag Health Study

Il1b: Interleukin 1 beta

IVC: Individually ventilated cages

KO: Knockout

LCFA: Long-chained fatty acids

LD: Lipid droplet

LF: Low fat

LFK: Low fat ketogenic control

LFS: Low fat-cholesterol-sucrose

Lipe: Lipase E, Hormone Sensitive Type

LPL: Lipoprotein lipase

Lxr: Liver X receptor

MCD: Methionine and choline deficient

MCDC: Methionine and choline deficient control

mRNA: messenger ribonucleotide acid

Na-Acetate: Sodium acetate

NaCl: Sodium chloride

NADH: Nicotinamide adenine dinucleotide

NAFLD: Non-alcoholic fatty liver disease

NASH: Non-alcoholic steatohepatitis

NEFA: Non-esterified fatty acids

PCR: Polymerase chain reaction

PkLr: Pyruvate kinase isozymes R/L

Pgc1: Peroxisome proliferator-activated receptor gamma coactivator 1

Plin: Perilipin

Pnpla2: Patatin Like Phospholipase Domain Containing 2

Polr2a: RNA Polymerase II Subunit A

Ppar: Peroxisome proliferator-activated receptor

Ppib: Peptidyl-prolyl cis-trans isomerase B

RIPA: Radio-immunoprecipitation assay buffer

RNA: Ribonucleic acid

Rpl32: Ribosomal Protein L32

Rplp0: Ribosomal Protein Lateral Stalk Subunit P0

RPM: Rotations per minute

RT: Reverse transcriptase

RT-qPCR: Quantitative reverse transcription polymerase chain reaction

Scd1: Stearoyl-Coenzyme A desaturase-1

SCFA: Short-chained fatty acids

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SEM: Standard error of the mean

Srebf1: Sterol Regulatory Element Binding Transcription Factor 1

TAG: Triacylglycerol

Tbp: TATA-Box Binding Protein

TBS-T: Tris buffered saline with Tween

Tnfα: Tumor necrosis factor-α

UCP1: Uncoupled protein 1

US: The United States of America

VLDL: Very low density lipoprotein

WAT: White adipose tissue

WT: Wild-type

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3 Introduction

3.1 Obesity

The proportion of people with obesity has increased over the last decades, especially in the US and Western world, and it is estimated that up to 58 % of men and 47 % of women are overweight or obese in Norway (1, 2). Data from the HUNT study in 2016 reported that a total of 76 % of men and 60 % of women in the age between 40-79 years were classified as either overweight or obese (3). Body mass index (BMI; kg/m^2), which is calculated by taking the body weight in kilograms and dividing it by height in meters squared (kg/m^2), is considered an easy and relatively reliable way to estimate obesity at a population level (4). A normal BMI is considered to range between 18.5kg/m^2 and 24.9kg/m^2, while a BMI from 25-29.9kg/m^2 is considered overweight, and a BMI > 30kg/m^2 is considered obese (2). BMI, however, does not take body composition into consideration, nor does it differentiate between lean and fat tissue or represent obesity directly, which affect health risks connected to excess weight gain (5). Waist-to-height ratio or waist circumference may be a better predictor of negative health effects, reduced life expectancy, and increased risk of comorbidities due to obesity, and gives especially a better estimate at the individual level (6). In 1980, the proportion of adults worldwide with a BMI greater than 25kg/m^2 was ~29 %, while it had increased to ~37 % in 2013. In Norway, a total of 66 % in the HUNT study were classified as overweight or obese in 2019 (2, 7). These numbers are still thought to rise, and it is estimated that up to 86 % of the adult population in the US will be either overweight or obese by 2030 (8).

Obesity, with central visceral adiposity, is one of the main risk factors for developing metabolic syndrome, in addition to increasing the risk for developing osteoarthritis (9), steatohepatitis (10), asthma (11-13), infertility (14, 15), diabetes, several types of cancer (16-19) and sleep apnoea (20). Additionally, excess bodyweight can reduce health-related quality of life (21, 22). The comorbidities following obesity is a major burden to the health care system and the overall economy. Obese individuals have 30 % higher medical costs compared to people with a normal weight (16). In US, a total of $113.9 billion was used to treat cases related to overweight and obesity, and account for a total of 5-10 % of the health care

spending (14, 16, 23). An estimate of 10 % of gross domestic product (GPD) in Norway is used on public health care, of which includes complications related to obesity, such as diabetes mellitus type 2 and cardiovascular complications (24). Finding a solution to the obesity epidemic and its comorbidities is an important challenge. If solved, it will reduce the economic burden for the society, in addition to improving public health and quality of life.

3.1.1 Non-alcoholic fatty liver disease

The increased prevalence in obesity has led to a parallel increase in non-alcoholic fatty liver disease (NAFLD), in which obesity is a major risk factor (25-27). NAFLD is estimated to affect between 25-50 % of people in different studies (with ethnic differences), and is considered the most common cause of chronic disease in the liver (27-30). Approximately 30 % of the overall adult American population is affected by NAFLD, while 36 % of the older population (40-79 years) in the HUNT study had NAFLD (7, 26, 28, 29). The amount of patients with NAFLD waiting for liver transplantation has increased from 2 % in 1994-95 to 6 % in 2011-2015 in the Nordic countries (31). People with NAFLD usually have an excess accumulation of triacylglycerol's (TAGs) in the hepatocytes (referred to as "steatohepatitis if the TAG content is over 5 % in the hepatocyte) (30, 32), which can later develop into nonalcoholic steatohepatitis (NASH) with the presence of injury of the hepatocytes, fibrosis, cell death and inflammation (28). NASH can further develop into cirrhosis, liver failure and hepatocellular carcinoma (27).

In contrast to alcoholic fatty liver disease, people with NAFLD usually develop the disease due to a caloric overconsumption, and are instead obese and often resistant to insulin (28). Other factors, such as hypertension, dyslipidemia, type 2 diabetes and metabolic syndrome are associated with a higher risk of developing NASH, in addition to a more progressive development of the disease (26). It is a challenge to screen for liver inflammation in individuals with NAFLD without biopsies, since most people with NAFLD are asymptomatic and liver blood tests may be normal (26, 27, 33). Ultrasounds or computed tomography may confirm fatty infiltration of the liver, and liver blood tests may be used to assess which individuals should be considered for a biopsy, but a biopsy is usually needed to confirm necroinflammation and liver injury (26, 33). The only current treatment of NAFLD is lifestyle and dietary interventions leading to a healthier diet, more exercise and weight loss (26, 28,

33-39). Some individuals with NASH may have an effect of bariatric surgery, which has shown to reverse NASH and fibrosis in some patients, but this is an option only for a minority of the patients, and the operation has its risk of complications (28, 38, 40). In the future, better prognostic and non-invasive techniques must be developed to be able to monitor the progression of NAFLD to NASH among patients, and to reduce the amount of people progressing from NASH to cirrhosis and liver failure (41, 42). With the obesity pandemic, more people will likely develop NAFLD, as obesity and metabolic syndrome are major risk factors for NAFLD, in addition to a higher risk of progression from NAFLD to NASH (26, 29, 33).

3.1.2 Chronic overfeeding and storage of fat

To maintain energy homeostasis and prevent weight gain, the body is dependent on a good balance between energy intake and energy expenditure. Excess energy is stored as neutral TAGs in lipid droplets (LDs) in the white adipose tissue (WAT) and other tissues (43-50). WAT can be found throughout the body, but are metabolically distinctly different in the visceral (fat around the central organs) and subcutaneous (abdomen, thighs and buttocks) areas of the organism (43). The white adipocytes are unilocular and store TAGs in one, single droplet, which constitutes more than 90% of the cell volume, and usually have a low number of mitochondria (43, 46, 51, 52). The LDs are surrounded by a phospholipid monolayer with several structural proteins, including perilipins (10, 53, 54). The white adipocytes are richly vascularized through loose connective tissue, which provides a delivery route for nutrients and oxygen (10, 47, 55). The adipocytes are also able to secrete both hormones (leptin) and cytokines, which are important for metabolic functions (10, 19, 47, 56-59). The WAT, in contrast to the brown adipose tissue (BAT), does not have the ability of thermogenesis through uncoupled respiration, which depend on UCP1 activity (60-62). Conversion of the metabolically inactive adipocytes found in WAT into more metabolically active adipocytes resembling those in BAT is considered an interesting possibility to prevent or reverse obesity.

In obese individuals, the amount of adipose tissue has expanded, and adipocytes in both omental and subcutaneous fat depots are increased in size (4, 63, 64). Increased accumulation of fat and expansion of WAT can lead to insulin resistance, hepatic steatosis, hypertension and other dysfunctions in the metabolism (10). For instance, hypertrophic adipocytes have

altered secretion of adipokines and cytokines, which affects insulin sensitivity (47, 65). This may also induce infiltration of macrophages in WAT and increase inflammation (19, 66-68). Obesity can also lead to accumulation of lipids as ectopic fat in non-adipose tissue, such as the liver and muscle (10, 41, 63, 69). Obesity is often associated with increased secretion of non-esterified fatty acids (NEFAs) from WAT resulting in increased influx of NEFAs to the liver (63). Insulin resistance in the WAT will result in a higher secretion of insulin from the pancreas. The secretion of insulin further stimulates lipid storage in the liver, and inhibits the β-oxidation of free fatty acids (FFAs) in the liver, resulting in steatosis (41). Over time, the steatosis in the liver can accumulate, resulting in the development of NAFLD.

3.2 The liver

3.2.1 Liver anatomy

The liver is the largest solid organ in the body, with a notorious anatomy and vascularity, and numerous tasks (70). The liver of an human adult weighs between 1-1.5 kg, and is positioned directly under the diaphragm, where it takes up most of the space (49). The liver has a pear-shaped organ placed immediately underneath the right side, which is the gallbladder. The liver receives its blood supply from two different sources: the hepatic artery and the hepatic portal vein (71, 72). The liver is distinguished for receiving the majority of its blood from a vein, which represents the liver's unique role in metabolism. The blood in the portal vein has already travelled through the blood system in the stomach and intestines, and carries substrates absorbed from the digestion of nutrients, which is delivered to the liver before they reach the general circulation (49). Another pair of important veins in the liver is the pancreatic veins, which joins the portal vein just before it enters the liver, and suffice hormones, such as insulin and glucagon, to the liver. These hormones wields their function on the liver before they reach the general circulation (49). The liver tissue is divided into lobules, which consist of hepatocytes neatly gathered in a hexagonal shape around the central vein, with portal triads consisting of branches from the portal vein, hepatic artery and the bile ducts at the very end from this hexagonal structure (73). The distinguished blood supply to the liver creates a gradient of oxygen and nutrients, which subsequently has led to the hepatocytes developing different tasks based on localization in the lobules (metabolic zonation) (73). In addition to

the hepatocytes, the liver contains several different non-hepatocyte cell types, including endothelial cells, Kuppffer cells, lymphocytes, biliary cells (producing bile, which is stored in the gallbladder) and stellate cells (72).

3.2.2 Metabolism in the liver

The liver is central in the metabolism of lipids and carbohydrates. Due to the anatomical placement of the liver, it is the first to "get its pick" from most digested nutrients (49, 74). Under fed conditions, where the access to nutrients are high and the body energy is in surplus, the portal vein will provide the liver with nutrients, such as glucose, branched chained amino acids (BCAAs) and short-chain fatty acids (SCFAs) from the digestive tract (74). The direct transportation of BCAAs to the hepatic portal vein, and the use of BCAA for treatment of liver diseases are considered an interesting research field (75-78). While SCFAs are absorbed directly to the portal vein, long-chained fatty acids (LCFAs) are incorporated into chylomicrons and secreted to the lymphatic system and the general circulation, before the remnants reach the liver (49, 73). The postprandial rise in glucose leads to increased secretion of insulin from the pancreas, which stimulates glycogenesis and *de novo* lipogenesis (73, 74, 79). The liver meets its energy requirements during these processes through active glycolysis (74).

Under fasted conditions, the glucose levels in the blood will subside gradually, leading to a drop in the secretion of insulin from the pancreatic β-cells, and instead lead to an increase in the secretion of glucagon. This results in a transition from glycolysis to gluconeogenesis in the liver for the production of glucose to provide fuel for the cells and the metabolic processes (80). The increase in the glucagon:insulin ratio will stimulate breakdown of the glycogen stored in the liver, as well as the β-oxidation of fatty acids to produce acetyl-CoA, which is later utilized in the Krebs cycle and oxidized to CO_2 (49). The energy, in the form of ATP produced in the Krebs cycle will be used to promote the transformation of glycerol, pyruvate or other three-carbon molecules into glucose in the gluconeogenesis (49). Excess acetyl-CoA stemming from oxidation of NEFAs secreted from adipose tissue is used to produce ketone bodies in the liver to provide energy to tissues in need.

3.3 Lipid metabolism

The liver is an essential organ for the coordination between the metabolism of both the carbohydrates and the lipids to ensure homeostasis of the energy requirements for the body, and is affected by the nutritional status and secretion of hormones (49, 81). The lipid metabolism includes production (*de novo* lipogenesis), esterification and storage of lipids, and breakdown of lipids (lipolysis and beta-oxidation).

3.3.1 Lipogenesis

When the intake of carbohydrates exceeds the requirements and the glycogen storage has met its capacity, the excess carbohydrates can be used for production of TAGs (82). During glycolysis, excess molecules of acetyl-CoA are made, which can be used for the synthesis of palmitic acid, known as *de novo* lipogenesis (49, 83). Glucose is the main substrate for de novo lipogenesis in liver, but other substrates, such as fructose, is also used when provided in excess (30, 83). Transcription factors such as sterol regulatory element-binding protein 1c (*Srebp1c*), liver x receptors (*LXR*s) and carbohydrate-responsive element-binding protein (*Chrebp*) are central for upregulation of lipogenesis under fed conditions, and these transcription factors are especially activated by the secretion of insulin, which activates kinases and phosphatases related to modification of these transcription factors (81). During fasting, these transcription factors can be phosphorylated and thereby inactivated to hinder the synthesis of fatty acids (81). Downstream genes, such as *Fasn* (synthesis of fatty acids), *Acc* (production of malonyl CoA) and *Scd1* (esterification of fatty acids) are all stimulated by these transcription factors and are important for the synthesis or desaturation of fatty acids (81). A balanced homeostasis between the activation and inactivation of genes and transcription factors related to lipogenesis is important, as an overexpression of central transcription factors have been shown to cause increased hepatic lipid accumulation and insulin resistance (81).

3.3.2 Lipid oxidation

When the access to nutrients are low and the body needs energy, TAGs stored in adipose LDs are mobilized to maintain energy homeostasis (45, 47-50). The released FAs enters the

circulation and is taken up by metabolically active organs, including liver. The nuclear receptor peroxisome proliferator-activated receptor alpha (*Pparα*) is central for the expression of genes involved in extramitochondrial and mitochondrial oxidation of FAs, and is central for regulation of hepatic lipid metabolism in liver, especially during fasting (84, 85). In addition, the hepatic expression of *Pparα* has been shown to be reduced in inflamed steatotic livers (85). To release energy and necessary substrates, the TAGs in the LDs are hydrolyzed to produce FAs and glycerol (for instance by ATGL which is encoded by the gene *Pnpla2* and HSL which is encoded by *Lipe*), which takes place in the cytoplasm (49, 86). The FAs taken up are activated to yield fatty acyl CoA, which binds to carnitine and gets transported to the mitochondrial membrane for β-oxidation. The fatty acyl CoA is then oxidized to acetyl-CoA, which enters the Krebs cycle to produce energy in the form of ATP (49). Enzymes such as Cpt1 and Acox1 are both central for lipid β-oxidation and are transcriptionally regulated by *Pparα* (85). If the Krebs cycle is over-stimulated by an excess amount of acetyl-CoA from the β-oxidation of FAs, the excess acetyl-CoA can be used for the production of ketone bodies instead, which can be used as fuel for organs, such as the brain if the access to glucose is low (49). Accumulation of fatty acyl CoA in the liver can be toxic, and the liver will prefer to route the fatty acyl CoA to produce TAGs and incorporate them in LDs, which can lead to NAFLD (49).

3.4 Lipid droplets

Lipid droplets are an efficient way of storing energy in the form of TAGs and cholesterol esters, in a core covered by a layer of phospholipids (such as phosphatidylserine and phosphatidylethanolamines) and several proteins, such as the perilipins (10, 87-89). This organization represent a high energy content in a very small volume, resulting in LDs acting as a major energy reservoir in many organisms. The amount of LDs must be balanced, as too few or too many LDs in various tissues results in different diseases, such as lipodystrophies in the case of lack of adipose tissue (10). The alteration in the basal homeostasis of LDs has also been associated with lipotoxicity (90). The LDs are formed from the endoplasmic reticulum (ER), where the enzymes necessary for catalyzing the synthesis of neutral LDs, such as ACATs for sterol esters and DGAT for TAGs, predominantly resides (10, 87). The phospholipids on the outer layer of the LDs give the droplets aqueous abilities, which is

important to prevent the coalescence of the LDs, which might contribute to diseases related to excess storage of lipids (10). Another important function of the LDs are the seclusion of lipid metabolites from the surrounding tissue, which minimize the toxic effects of a plethora of FFAs or sterols, which can lead to inflammation and insulin resistance (88, 91, 92). The LDs are also involved in processes such as cell signaling and trafficking of vesicles (93). As mentioned earlier, the surface of LDs are usually covered with perilipin proteins, which is important for the regulation of the LDs, including size (94).

3.5 Perilipin proteins

The various perilipin proteins have a distinguished tissue distribution, which may suggest individual specialization and tasks among the Plins (94). The different Plins are highly maintained between different species, which strengthens their importance regarding the regulation of metabolism and distribution of the LDs (90, 93-95). To date, five different perilipin genes have been identified. Perilipin 1-4 is especially connected to the regulation and number of the LDs, while Plin5 is important for the regulation of lipolysis in hepatocytes (93, 94).

3.5.1 Plin1

Plin1 was the first perilipin protein to be discovered, with Plin1a being the most abundant in adipocytes in WAT and BAT, while Plin1c is found in steroidogenic cells (88, 94). Plin1 is also weakly expressed in human hepatocytes of individuals with fatty liver (10, 96, 97). Plin1 is important for inhibition of lipolysis in adipocytes in the basal state, and may have a protective role against inflammation and insulin resistance in adipose tissue (88). Plin1 has been suggested to have an important role in the regulation and function of adipose tissue, since inactivation of Plin1 in mice resulted in leaky LDs and partial lipodystrophy (93, 98-100).

3.5.2 Plin2

In contrary to Plin1, Plin2 coat LDs in various cell types and is important for accumulation of lipids and stability of the formed LDs (86, 90). Expression of Plin2 has been shown to protect

the LDs against autophagy, which results in increased accumulation of LDs (101). An increase in the gene expression of *Plin2* has been associated with atherosclerotic plaques, with a lack of gene expression of *Plin2* associated with a reduction in the formation of atherosclerotic plaques in mouse models of atherosclerosis (102). Compared with controls, an overexpression of Plin2 have been showed to suppress lipolysis, and thus increase cellular levels of TAGs and the size of LDs (94, 103). Plin2 has been hypothesized to increase the uptake of LCFAs, in addition to inhibit the β-oxidation in liver (102). High levels of Plin2 in humans have been associated with diseases such as hepatic steatosis, sarcopenia, atherosclerosis and cancer (90, 101).

3.5.3 Plin3

Plin3 has the same distribution as Plin2, and has numerous tasks. Expression of Plin3 increases in the small intestine after an intake of bolus with dietary fat, where it packs and transport the dietary lipids in an efficient manner (90, 104). Plin3 has also been shown to increase in muscles after endurance exercises among healthy people, where Plin3 may have an influence on lipid oxidation (90, 105). In Plin2$^{-/-}$ KO mice, both Plin3 and Plin5 will increase as a compensation for the loss of Plin2, and this switch has been hypothesized to protect against steatosis in the liver (94).

3.5.4 Plin4

Plin4 can be found primarily in white adipocytes, with some distribution in organs such as brain, heart, and skeletal muscle, and is known to be regulated by PPARγ (93, 94). The clear physiological function of Plin4 in the development of adipose tissue is unknown, but a loss of Plin4 leads to a downregulation of Plin5, in addition to a decreased accumulation of lipids in the heart under basal conditions (93). A loss of Plin4 expression in the heart together with the reduced expression of Plin5 have been proposed to be protective against genetically and dietary induced cardiac steatosis (93).

3.5.5 Plin5

Plin5 is highly expressed in fatty acid oxidizing tissues, such as BAT, skeletal muscles and heart (94). In liver, heart and skeletal muscles, Plin5 is activated by PPARα, while it is regulated by PPARγ in adipose tissue (93, 106). Plin5 is also influenced by the expression of Plin4, since a loss of Plin4 will lead to downregulation of Plin5 in several tissues, especially in the heart (93). Plin5 is connected to downregulation of lipolysis and fatty acid oxidation in liver cells, which leads to accumulation of TAGs (41, 107). The expression of Plin5 increases in individuals with steatotic livers, while a deficiency of Plin5 have been shown to reduce the lipid contents in the liver, in addition to a reduction in the size of the LDs (41, 108).

3.6 Aim and hypothesis

The overall aim of this study was to investigate how diets with different content of carbohydrates, lipids, methionine and choline or energy deficiency (fasting) affects accumulation of lipids and transcriptional regulation of genes involved in energy metabolism in the liver. The hypothesis was that diets with a high content of saturated fat and sucrose would increase bodyweight, fat mass and steatosis in liver. A high content of fats and low content of carbohydrates was hypothesized to increase expression of genes related to lipid oxidation and decrease expression of genes related to lipogenesis. A diet lacking the essential amino acids methionine and choline was anticipated to lead to inflammation and liver damage. All diets were expected to increase lipid accumulation in liver, but to various extent.

The specific aims for this master thesis were to:

- Analyse weight gain for the various diets.
- Analyse changes in body composition, such as amount of fat mass, liver weight and liver weight relative to body weight.
- Analyse accumulation of TAG in the livers and the abundance of Plin2 and 3.
- Analyse mRNA expression of genes related to LD binding, lipolysis, lipogenesis, and inflammation, as well as central transcription factors.

4 Material and Methods

4.1 Material

4.1.1 Material used for data collection and molecular analyses

Table 4.1. Equipment. Equipment used for molecular analyses and data collection.

Equipment	Producer
ABI Prism 96-Well Optical Reaction Plate	Applied Biosystems (Foster City, US)
Bioruptor Plus® sonication device	Diagenode (Liège, Belgium)
Bioruptor® Water Cooler	Diagenode (Liège, Belgium)
Centrifuge, Biofuge fresco	TermoFisher Scientific (Waltham, MA, US)
Centrifuge, Mini Spi, Eppendorf	Eppendorf AG (Hamburg, Germany)
Centrifuge, Mini Spi, PCR Strips	TermoFisher Scientific (Waltham, MA, US)
CFX96 Real-Time System Thermal Cycler	Bio-Rad Laboratories (Hercules, CA, US)
ChemiDoc™ Touch Imaging System	Bio-Rad Laboratories (Hercules, CA, US)
Criterion™ Cell system	Bio-Rad Laboratories (Hercules, CA, US)
Criterion™ TGX™ Gel, 26 Well Comb, 15 µl, 4-20%	Bio-Rad Laboratories (Hercules, CA, US)
Criterion™ TGX™ Gel, 26 Well Comb, 15 µl, 10%	Bio-Rad Laboratories (Hercules, CA, US)
Eppendorf Research Plus Pipettes	Eppendorf AG (Hamburg, Germany)
Eppendorf tubes	Sarstedt (Germany)
Falcon tubes	Falcon, Corning Incorporation (Durham, US)
Filter paper	Bio-Rad Laboratories (Hercules, CA, US)
Glass beads (1 mm)	Glass beads, Assistant, (Germany)
Gloves	Kimtech, Kimberly-Clark Professional
Mastercycler Ep Gradient S	Eppendorf AG (Hamburg, Germany)
Microseal, B'Adhesive Seals for PCR Plates	Bio-Rad Laboratories (Hercules, CA, US)
Microtest Plate 96 Well F	Sarstedt (Germany)
Micro tubes	Sarstedt (Germany)
Multi-channel pipette	Eppendorf Research (Germany)
Nano Drop ND-1000 Spectrophotometer	TermoFisher Scientific (Waltham, MA, US)
PCR strips with attached caps	TermoFisher Scientific (Waltham, MA, US)
Pipette tips, Biosphere (5mL, 10mL)	Falcon, Corning Incorporation (Durham, US)
Pipette tips (0.5-10 µl, 0.1-20 µl, 2-100 µl, 2-200 µl,1250 µl)	Eppendorf Research Plus (Hamburg, Germany)
Pipetboy acu 2	Integra Bioscience (Hudson, US)
PowerPac™ Basic	Bio-Rad Laboratories (Hercules, CA, US)
PreLys24 Homogenizer	Bertin Instruments (France)
Synergy H1™ Hybrid Multi-Mode Reader	BioTek (Winooski, VT, US)
Trans-Blot® Turbo™ Transfer System	Bio-Rad Laboratories (Hercules, CA, US)
Well plate (12 wells)	Sigma-Aldrich (St. Louis, MO, US)

Table 4.2 Chemicals and reagents. Chemicals and reagents used for molecular analyses and data collection in this master thesis.

Chemicals and reagents	**Producer**
Beta-mercaptoethanol	Sigma-Aldrich (St. Louis, MO, US)
Bovine serum albumin (BSA)	Sigma-Aldrich (St. Louis, MO, US)
Bromophenol blue sodium salt	Sigma-Aldrich (St. Louis, MO, US)
Chow diet	Research Diets INC (US)
DL-Dithiothreitol	Sigma-Aldrich (St. Louis, MO, US)
Donkey Anti-Guinea Pig IgG (H+L)	Jackson ImmunoResearch (PA, US)
Dry ice	Praxair (Guildford, UK)
EDTA	Sigma-Aldrich (St. Louis, MO, US)
Ethanol (EtOH) 96%	Sigma-Aldrich (St. Louis, MO, US)
Glycerol (>99.0%)	Sigma-Aldrich (St. Louis, MO, US)
Glycine	Sigma-Aldrich (St. Louis, MO, US)
Goat Anti-Rabbit IgG	Jackson ImmunoResearch (PA, US)
Hydrochloric acid	Sigma-Aldrich (St. Louis, MO, US)
High fat control diet (low-fat diet)	Research Diets INC (US)
High fat diet	Research Diets INC (US)
Ketogenic control diet	Research Diets INC (US)
Ketogenic diet	Research Diets INC (US)
Methanol (> 99.9%)	Sigma-Aldrich (St. Louis, MO, US)
Methionine and choline control diet	Research Diets INC (US)
Methionine and choline deficient diet	Research Diets INC
Milli-Q water	Millipore (Massachusetts, US)
Nonidet™ P 40, 100%	Sigma-Aldrich (St. Louis, MO, US)
PCR-grade water	Sigma-Aldrich (St. Louis, MO, US)
Perilipin 2 (N-terminus aa 1-29), Rabbit	Progen (Heidelberg, Germany)
Perilipin 3 (mouse C-Terminus), Guinea pig	Progen (Heidelberg, Germany)
Phenol:Chloroform:Isoamylalcohol (24:24:1)	Invitrogen (Life technologies, Paisley, UK)
Phosphate-buffered saline (PBS)	Sigma-Aldrich (St. Louis, MO, US)
Phosphatase inhibitor	Sigma-Aldrich (St. Louis, MO, US)
Precision Plus Protein™ Standards (All Blue)	Bio-Rad Laboratories (Hercules, CA, US)
Precision Plus Protein™ Standards (Dual Color)	Bio-Rad Laboratories (Hercules, CA, US)
Primers (*see appendix 1*)	Sigma-Aldrich (St. Louis, MO, US)
Protease inhibitor	Boehringer Mannheim
RA1 Lysis Buffer	Macherey-Nagel (Düren, Germany)
Sample buffer, Laemmli (4x concentrate)	Sigma-Aldrich (St. Louis, MO, US)
Sodium chloride, NaCl ((Physiological concentration of 150 mM)	Sigma-Aldrich (St. Louis, MO, US)
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich (St. Louis, MO, US)
Sodium deoxycholate	Sigma-Aldrich (St. Louis, MO, US)
Sodium chloride (NaCl)	Sigma-Aldrich (St. Louis, MO, US)
SsoAdvanced Universal SYBR Green Supermix	Bio-Rad Laboratories (Hercules, CA, US)
SuperSignal® West Pico Chemiluminiscent Substrate	TermoFisher Scientific (Waltham, MA, US)
Tris base	Sigma-Aldrich (St. Louis, MO, US)
Tris-HCl	Sigma-Aldrich (St. Louis, MO, US)
Tween® 20	Sigma-Aldrich (St. Louis, MO, US)
Western diet	Research Diets INC (US)
Western control diet	Research Diets INC (US)

Table 4.3. Kits. All kits used for molecular analyses and data collection in this master thesis.

Kit	Producer
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems (Warrington, UK)
NucleoSpin RNA kit	Macherey-Nagel (Düren, Germany)
Pierce™ BCA Protein Assay Kit	TermoFisher Scientific (Waltham, MA, US)
Triglycerides Enzymatique PAP 150 (TG PAP 150)	BIOLABO SA (Maizy, France)
Trans-Blot® Turbo™ RTA Transfer Kit, Nitrocellulose (Midi-size)	Bio-Rad Laboratories (Hercules, CA, US)

Table 4.4. Internet resources and software programs. Internet resources and software programs used for collection, handling and processing of data in this master thesis.

Program	Producer
EndNote X9.2	Thomson Reuters
Gen5 Microplate Reader and Imager Software v3.02	BioTek (Winooski, VT, US)
GraphPad Software	GraphPad Software Inc. (San Diego, CA, US)
Image J 1.50i	National Institutes of Health , USA
Image Lab™ Software	Bio-Rad Laboratories (Hercules, CA, US)
Microsoft Office 2016	Microsoft®
ND-1000 Software	Saveen & Werner AB, Sweden
7900 SDS v2.3	TermoFisher Scientific (Waltham, MA, US)

4.2 Methods

4.2.1 Ethical considerations

All care and use of the mice in this master thesis, in addition to all the procedures performed with them, were in accordance with the guidelines for care and use of experimental animals in Directive 2010/63/EU of the European Parliament, which describes the correct care and use of animals in scientific experiments. All mice were housed in individually ventilated cages (IVC) and had accommodations with three to four other animals in an environment with controlled temperature of 22°C and a strict 12 hours light/dark cycle.

4.2.2 Animal models

The lipid droplet research group use genetically altered mice as models to understand the biological role of perilipin proteins. A number of diet interventions have been performed in colonies of Plin4 mice (Plin4$^{+/+}$ and Plin4$^{-/-}$ mice) and Plin5 mice (Plin5$^{+/+}$ and Plin5$^{-/-}$ mice). All animals have been back-crossed into the C57BL/6N strain for a minimum of ten

generations. In this master thesis, female wild type (WT) mice (Plin4$^{+/+}$ and Plin5$^{+/+}$) given various diets have been included in the analysis. These control mice are genetically unaltered and isogenic, and will be referred to as C57BL/6N mice. All animal work and sampling of tissue was conducted before the start of this master project.

4.2.3 Diet interventions

A total of 89 WT mice were used in five different, independent diet intervention experiments. The mice were divided into an intervention group and a control group in each experiment, resulting in 10 different groups. The control and the intervention groups of each study were matched in age. Before the intervention, the mice were given free access to chow food (containing 58E% carbohydrate, 18E% fat and 24E% protein) and water (diets from Envigo, Indiana, USA).The mice were then either fasted or exposed to custom-made diets (Research Diets Inc, USA), as summarized in table 4.5. Each mouse was euthanized by cervical dislocation at the end of the diet intervention, and bodyweight was measured. The organs were sampled and weighted (liver), while the amount of fat were measured (subcutaneous fat pads in the inguinal region and gonadal fat mass) and frozen in liquid nitrogen and stored at -80°C. In this master thesis, bodyweight, liver tissue, protein and gene expression have been analysed.

The diets used in the study were the following:

1. Standard rodent chow diet or 24 hours fasting at 15 weeks of age.
2. Normal access to a low fat-cholesterol-sucrose control diet or a high fat-cholesterol-sucrose diet (a diet high in fat/sucrose/cholesterol) for 12 weeks, from the age of 8 weeks to ~20 weeks
3. Normal access to a low fat control diet, or a high-fat diet for 12 weeks, from the age of 8 weeks to the age of ~20 weeks
4. Normal access to a low fat ketogenic control diet (same as HFD control diet) or a high fat ketogenic diet for 4 weeks, from the age of ~16 weeks to ~20 weeks
5. Normal access to a methionine and choline deficient control diet or a methionine and choline deficient diet for 4 weeks, from the age of 12 weeks to the age of ~16 weeks

Table 4.5. Diet compositions and rodents included in this study. The mice were fed *ad libitum* with regular chow diet, fasted for 24 hours (fasted) or fed 7 different custom-made diets (Research Diets, Inc); The diets used differed in the content of methionine and choline or the composition of carbohydrates and lipids. The diets used were: Regular chow diet (**chow**), low fat-cholesterol-sucrose diet (**LFS**), high fat-cholesterol-sucrose diet (**HFS**), low fat diet (**LF**), high fat diet (**HF**), low fat ketogenic control diet (**LFK**), high fat ketogenic diet (**HFK**), methionine and choline deficient control diet (**MCDC**), and methionine and choline deficient diet (**MCD**). The number of mice receiving the different diets are indicated on the right. The LF and LFK mice received the same diet.

Diet intervention	Nutrient composition (of total)			Diet composition, calories in %			# animals	Kcal/g
	Chol (mg)	**Sucrose**	**Saturated Fat**	**Protein**	**Carb**	**Fat**		
Chow	-	-	0.9%	24%	58%	18%	9	3.1
Fasted	-	-	-	-	-	-	10	-
LFS	-	-	3 %	20 %	60 %	20 %	8	4.0
HFS	10600	32 %	10 %	20 %	40 %	40 %	8	4.5
LF	54	35 %	2.2 %	20 %	70 %	10 %	10	3.85
HF	216	6.8 %	18 %	20 %	20 %	60 %	12	5.24
LFK	54	35 %	2.2 %	20 %	70 %	10 %	8	3.85
HFK	325*	-	25 %	20 %	0.1 %	80 %	8	6.14
MCDC	-	43 %	13 %	16 %	63 %	21 %	8	4.2
MCD	-	43 %	13 %	16 %	63 %	21 %	8	4.2

*** Estimated cholesterol level.**

4.2.4 Quantitative reverse transcription polymerase chain reaction

To assess gene expression, the method quantitative reverse transcription quantitative polymerase chain reaction (RT-qPCR) was chosen. In this method, ribonucleic acid (RNA) is isolated, reverse transcribed into complementary deoxyribonucleic acid (cDNA), and finally the levels of specific cDNA are determined with quantitative PCR.

Tissue homogenization and RNA isolation

Small pieces of liver tissue (~5mg) were added to a tube containing 0.5mL RA1 buffer (Machery-Nagel, Düren, Germany) containing 1% β-mercaptoethanol (3.5 µL β-mercaptoethanol in 350 µL RA1 buffer) and glass beads. The tissue was rapidly homogenized for 2 x 30 s at 5000 revolutions per minute (rpm) in a PreLys24 homogenizer (Bertin instruments). Total RNA was subsequently isolated from liver tissue as described in the NucleoSpin RNA kit manual (Macherey-Nagel) with two minor alterations prior to application to the purification column. After homogenization, 350 µL of the Phenol-Chloroform-Isoamylalcohol-solution was added to each sample in a fume hood, followed by

vigorous shaking for 20 seconds. All samples were left in room temperature for two minutes. This was followed by centrifuging the samples at 9000 rpm for five minutes. A total of 300 µL of the supernatant from each sample was transferred to a new Eppendorf tube in a fume hood. A volume of 80 µL of a high salt solution with the concentrations 1.5 M NaCl and 0.8 M Na-Acetate (pH 5.5) was added to each sample before mixing comprehensively by hand. After mixing, 225 µL of 96% ethanol was added and the samples were mixed again. Total sample volume (~605 µL) was passed over to the Nucleospin purification columns for further processing according to the manufacturer`s instructions.

Determination of RNA concentration

The concentration and quality of the RNA was determined by measures of absorbance with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) following a protocol previously described (109). Absorbance was measured at 260nm to determine the RNA concentration. Determination of RNA quality was based on the following absorbance ratios: 260 nm/280 nm should be 2.0 (lower levels indicates protein contamination) and 260 nm/230 nm should be around 2.2 (lower levels indicates phenol or guanidine contamination). Ratios >1.9 was considered acceptable and the RNA sample was processed further.

Synthesis of cDNA

First strand cDNA was made by utilizing the RNA previously extracted, and reverse-transcribe it with multiscribe reverse transcriptase (RT) enzyme with random primers, according to the protocol described in the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). This protocol is analogous to the protocol described by Haddad *et. Al* (110). The constituents in the master-mixture are summarized in table 4.6.

Table 4.6. Constituents in the reverse transcriptase master-mix. RT: reverse transcriptase; dNTP: deoxynucleoside triphosphate; cDNA: complementary deoxyribonucleic acid; mRNA: messenger ribonucleotide acid.

Reagents	**Volume in each tube (µL)**	**Function of reagent**
RNAse free H2O	2.1	Used for dilution
10x RT buffer	1	Used for the regulation of pH
10x Random primers	1	Primers for cDNA synthesis
dNTP mix	0.4	Building blocks for the cDNA
Multiscribe RT enzyme	0.5	Enzyme for conversion of mRNA into single stranded cDNA
Total volume	**5**	

To simplify pipetting and minimize pipetting errors, all RNA samples were carefully diluted to a common concentration of 100 ng/µL before the cDNA synthesis was started. A total of 5 µL master-mixture and 5 µL of diluted RNA made a total reaction volume of 10 µL. In addition to the samples, two controls were made for each independent diet intervention study. These controls consisted of PCR water, which were used to confirm no contamination in the samples, and "no-RT", which were a mix of three randomly picked samples for each diet intervention study lacking the reverse transcriptase enzyme, to confirm no contamination of DNA in the samples. A thermocycler (Mastercycle Ep Gradient S, Eppendorf AG (Hamburg, Germany)) was utilized in the cDNA synthesis. The settings used were as followed; Annealing: 10 minutes at 25°C (for the primers to bind to the RNA); Reverse transcription: 120 minutes at 37°C (for the reverse transcriptase enzyme to transcribe cDNA with RNA as the template); Enzyme inactivation: 5 minutes at 85°C (to inactivate the reverse transcriptase enzyme). A total of 10 µL cDNA was diluted in a tube with 90 µL RNAse free H_2O to achieve accurate pipetting and optimize the results from the RT-qPCR analysis. The final cDNA concentration was 5 ng/µL.

Real time quantitative PCR

cDNA (2.5µL) and RT-PCR master mix (7.5µL ; table 4.7) was pipetted into a 96 well plate, giving a total reaction volume of 10µL. Each gene studied required a specific primer pair (appendix 1), which resulted in one master mix for each gene assay. The fluorescent dye SYBR Green (SsoAdvanced, Bio-Rad) intercalates in double stranded DNA and reflects the amount of double stranded DNA in sample. The dye was added to detect the amplified

product of the PCR reaction. In addition to the samples, the two controls made during the cDNA synthesis were pipetted as well for each primer sample set.

Table 4.7. Constituents in the master-mix for RT-qPCR. SYBR Green was chosen as the fluorescent dye to detect the amount of double stranded DNA amplified in the RT-qPCR assay (the final product). To investigate different genes, specific primer pairs (5' and 3') were utilized (appendix 1). DNA: deoxyribonucleic acid; RT-qPCR: Quantitative reverse transcription polymerase chain reaction.

Components	Reagents volume (1 reaction)
SYBR Green mix	5 µL
PCR H_2O	2.3 µL
5'primer (10 µM)	0.1 µL
3'primer (10 µM)	0.1 µL

The instrument CFX96 Real-Time System Thermal Cycler (Bio-Rad Laboratories (Hercules, CA, US)) and the SDS 2.3 software (Applied Biosystems) were utilized to conduct the qPCR amplifications. The cyclic conditions used involved a step with denaturation (three minutes at 95°C), followed by a cycle consisting of 10 seconds at 95°C, thereby 20 seconds at 60°C, which were repeated 40 times. The fluorescence was measured at the end of each cycle. The relative mRNA levels of gene expression were quantified by utilizing the comparative ΔΔCT model (111). Several common reference genes were tested to identify a reference gene that were unaltered by fasting or the different diets. Based on these analyses, the Rpl32 gene was selected as the most optimal reference gene for gene expression analysis in liver.

4.2.5 Measurement of hepatic content of lipids

Pieces of liver tissue (~20mg) were cut and weighed and transferred to 2mL tubes with screw cap and glass beads for homogenization. A total of 100 µL 1xRIPA lysis buffer were added for each 10mg of liver tissue before the tissue were homogenized in a PreLys24 homogenizer (Bertin Instruments) with shaking for 2 x 30 s at 6000 rpm. The samples were then sonicated with a Branson Sonifier 450 for 3x 30s with the highest setting. An aliquot of 5 µL from each sample was transferred to a new Eppendorf tube and diluted 32 times with 160 µL Milli-Q water. The rest of the samples were stored for later analyses. The quantification of triglycerides were measured with the Triglycerides Enzymatique PAP 150 kit (BIOLABO). A 7-point standard dilution curve was made for comparison with the concentrations 200, 100, 50, 25, 12.5, 6.25 and 3.125 mg/dL. A total of 16 µL standard with 1 µL 0.5xRIPA was added

to each well. The diluted samples were vortexed before 16 µL sample were pipetted to the plate. A total of 200 µL of the triglyceride reagent (BIOLABO SA) at room temperature was added to each well. The contents of the plate were mixed and incubated at 37°C for five minutes and then read at 500 nm in the Synergy H1™ Hybrid Multi-Mode Reader (BioTek). The total triglyceride content was calculated relative to the standard dilution curve.

4.2.6 Western blotting

The small pieces of liver isolated for the use of measuring triglyceride contents were also used for denatured Western blotting by utilizing electrophoresis (SDS-PAGE).

Protein measurement and pooling of samples

A BCA assay (ThermoFisher Scientific, MA, US) was used for measuring protein concentration of each sample. An aliquot of 50 µL sample was taken from each tube and centrifuged at 12000 rpm for 20 minutes to spin down debri. The clear fraction was pipetted to a new tube and diluted 25 times in MilliQ H_2O. Bovine serum albumin (Sigma-Aldrich, MO, US) was used to make a 7-point standard dilution curve consisting of concentrations of 2000, 1000, 500, 125, 62.5, 31.25 and 0 µg/µL for comparison. A total of 12.5 µL of the diluted samples and the standard curve were transferred to each well in a 96-well plate. The colorimetric reagent was mixed in a 50:1 ratio and a total of 200 µL were added to each well, before the samples were incubated at 37°C for 30 minutes and absorbance measured at 562 nm in the Synergy H1™ Hybrid Multi-Mode Reader (BioTek). The total protein content was calculated in Excel by utilizing the standard dilution curve. The samples were then diluted to achieve a common concentration of 4.4 µg/µL protein based on the sample with the lowest reading at 562nm. An aliquot of 5 µL was taken from each sample from each group in the diet interventions and pooled into new tubes for further procedures.

Gel-electrophoresis and blotting

A 37.5 µL aliquot of each pooled sample was mixed with 12.5 µL of 4x Laemmli (Sigma-Aldrich, MO, US) with DTT. The samples were then heated for five minutes at 95°C.The SDS-PAGE was done using the Criterion™ Cell system with Criterion TGX Precast gels with

4-20% or 10% acrylamide (Bio-Rad Laboratories, CA, US). The gels were placed and locked in position in the Criterion™ Cell system, and running buffer was added to the chambers (100mL 10x running buffer, 900mL dH_2O). Each well in the gels were washed using a pipette and running buffer. Precision Plus Protein™ Standards ("All Blue" and "Dual Color") were added to the first and last well in the gels, in addition to 10 µL sample in duplicates in the remaining wells. The gels were then run at 190 V for about 45 minutes until the samples reached the bottom of the wells. The proteins in the gels from the electrophoresis were then transferred to a nitrocellulose membrane by using the "Trans-Blot® Turbo™ RTA Transfer Kit, Nitrocellulose" and the "Trans-Blot® Turbo™ Transfer System" from Bio-Rad Laboratories (Bio-Rad Laboratories, CA, US) as described in the manufacturer's instructions. Briefly, the membranes and transfer stacks were equilibrated in transfer buffer. Transfer stacks, membrane and gel were assembled in the transfer cassette, and transferred for 7 minutes at 2.5A. The membranes were then stained with Ponceau S to enable quantification of total protein in each lane (112, 113).

Immunodetection

The membranes were incubated in blocking buffer (5% BSA in TBS-T) in room temperature for 60 minutes while being tilted slowly. The membranes were then incubated with the primary antibody (either rabbit anti-Plin2 (catalogue #nb110-40877) or guinea pig anti-Plin3 (catalogue #RDI-progp 30), diluted 1:1000 in 2.5% BSA in TBS-T) over night at 4°C. The next day, membranes were washed in TBS-T four times for five minutes. The membranes were then incubated with the HRP-conjugated secondary antibodies (goat anti-rabbit IgG (catalogue #4050-05) or donkey anti-guinea pig IgG (catalogue #706-035-148), diluted 1:10000 in TBS-T with 5% BSA) for 60 minutes while being tilted slowly. The membranes were then rinsed in TBS-T and washed three times for five minutes. For chemiluminescence detection, the membranes were incubated with SuperSignal® West Pico Chemiluminiscent Substrate (Termo-Fisher Scientific, MA, US), and emitted light was captured using the ChemiDoc™ Touch Imaging System (Bio-Rad Laboratories, CA, US). Band intensities were calculated in ImageJ by measuring the intensity of each peak, and subtracting background intensity.

4.2.7 Statistical methods

All the data presented in this master thesis have been analyzed using Excel (Microsoft®) and GraphPad Prism for Microsoft (GraphPad Software, San Diego, CA, US). The data are presented as means with standard error of the mean (SEM). The results of bodyweight, body composition, amount of liver TAG and protein and gene expression from the different diets were analyzed by utilizing a student's t-test, which reveals if the mean from one independent group differs significantly from the other independent group. The t-test was used to check for significant differences between the control group and the diet intervention group for each individual experiment. Since the fed/fasting experiment and the four diet interventions were independent experiments, no statistical evaluation was performed between the groups from different diet interventions. The level of significance was set at 0.05, and a significant difference was marked with a "*" in the figures and tables.

5 Results

In this master thesis, data collected *in vivo* and biological material from female C57BL/6N mice exposed to various diet interventions have been analysed. The mice included in the analysis had *ad libitum* access to diets where the amount or composition of carbohydrates, lipids, cholesterol, methionine and choline had been altered, whereas a last group of animals has been fasted for 24 hours. Total body and organ weights, as well as hepatic TAG content, selected mRNA and protein expression have been analysed. A total of 89 mice were included in the analysis. Animals were included from five separate diet interventions that were completed prior to this project. All data presented in this master thesis were analysed and/or generated in this master project. Although data from all interventions are presented in the same graphs, data analysis are restricted to comparing the two groups pr. intervention (marked as control [C] and diet intervention [D]).

5.1 Mouse characteristics

The content of carbohydrate, lipids, cholesterol, methionine and choline in the diets used is described in the material and methods section (Table 4.5). The various diets affected bodyweight and liver and adipose tissue weights differently (figure 5.1). As expected, mice lost fat, liver and body mass after 24 hours of fasting. The mice fed the diet high in fat, sucrose and cholesterol (HFS) had a higher bodyweight, liver weight and liver mass compared to mice fed the diet low in fat, sucrose and cholesterol (LFS). Little difference was seen between mice receiving low fat (LF) versus high fat (HF) diets, except for increased adipose tissue fat mass in the inguinal area. A small decrease in liver weight was observed for mice receiving the high fat ketogenic (HFK) diet compared to controls. Mice fed the methionine and choline restricted diet (MCD) had increased relative liver weight and lost substantial amounts of fat in the gonadal and inguinal areas compared to mice receiving methionine and choline control diet (MCDC). The age of the animals included in the analysis were 15 weeks or 20 weeks at completion of the diet interventions. No significant differences were observed between the controls and the mice in the intervention groups for the remaining diets.

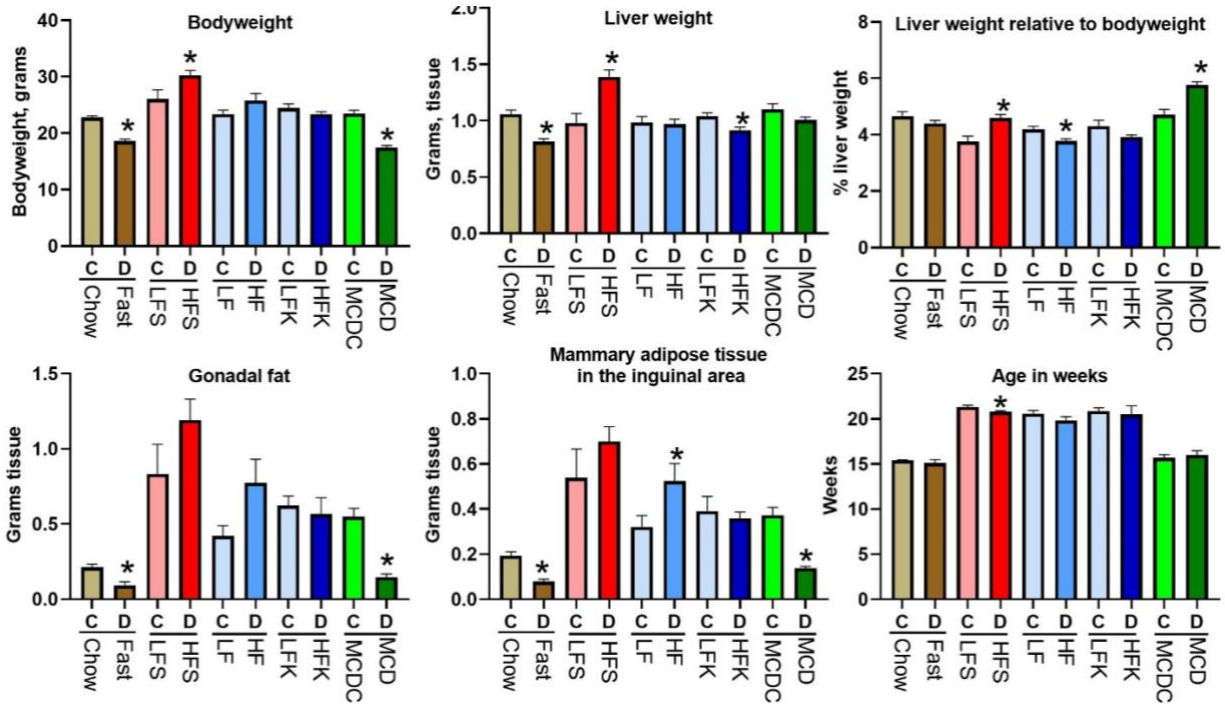

Figure 5.1. Impact of the diets on bodyweight, liver weight and adipose tissue. Female C57BL/6N mice from 5 independent animal studies were included. Mice received chow diet, were fasted for 24 hours, or received diets with variable content and/or composition of carbohydrates, lipids, cholesterol, methionine and choline. The stars indicate significant difference between the control group (C) and the diet intervention group (D) for each individual experiment (p-value < 0.05). Abbreviations used: C: control group; D: diet group; chow (n=9); fast (n=10); LFS: low fat-cholesterol-sucrose diet (n=8); HFS: high fat-cholesterol-sucrose diet (n=8); LF: low fat diet (n=10); HF: high fat diet (n=12); LFK: low fat ketogenic control diet (n=8); HFK: high fat ketogenic diet (n=8); MCDC: methionine and choline deficient control diet (n=8); MCD: methionine and choline deficient diet (n=8).

5.2 Triglyceride contents and Plin expression

To determine hepatic lipid accumulation with the various diets, the amount of TAG in the liver was measured (figure 5.2). Mice that were fasted or received the HFS or the MCD diets had a higher accumulation of TAG in the liver compared to controls. The content of TAG was unaltered for the HF and the HFK diets compared to their control diets. As an alternative measurement of lipid accumulation, the protein expression of LD coating proteins Plin2 and Plin3 was measured with immunoblotting. Expression of Plin2 seemed to increase with fasting, as well as with all dietary interventions compared to the respective control diets (figure 5.3). Expression of Plin3 seemed to increase with HF, HFK and MCD diets, compared to their control diets. There was in general an overlap in TAG levels and expression of Plin2 or Plin3 proteins, with the exceptions of high Plin2 and Plin3 expression but low TAG levels in the HF and HFK groups.

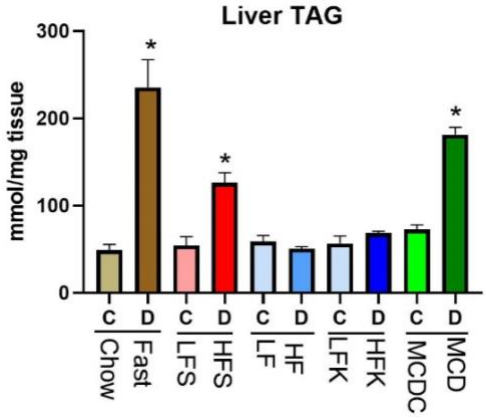

Figure 5.2. The amount of TAG in the livers of the mice from the different diet interventions. The amount of TAG are shown in mmol/mg tissue. Significant differences between diet and control groups are marked with stars (p-value<0.05). Abbreviations used: C: control group; D: diet group; chow (n=9); fast (n=10); LFS: low fat-cholesterol-sucrose diet (n=8); HFS: high fat-cholesterol-sucrose diet (n=8); LF: low fat diet (n=10); HF: high fat diet (n=12); LFK: low fat ketogenic control diet (n=8); HFK: high fat ketogenic diet (n=8); MCDC: methionine and choline deficient control diet (n=8); MCD: methionine and choline deficient diet (n=8).

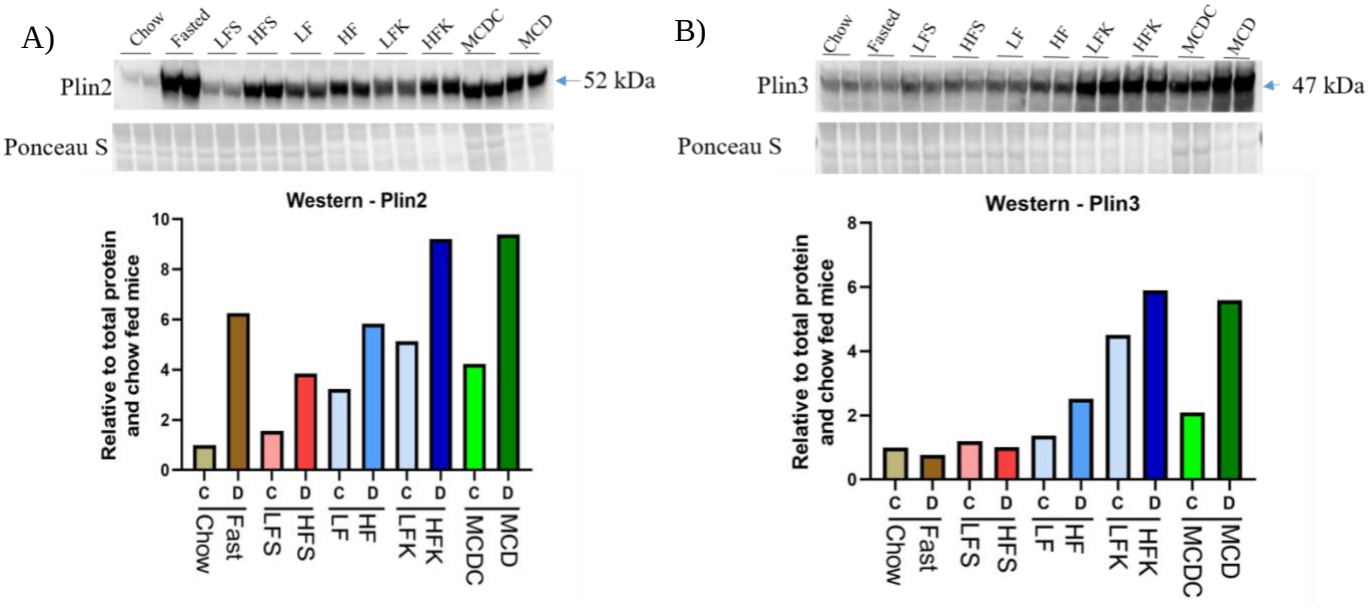

Figure 5.3. Relative protein expression of Plin2 and Plin3 in the liver of different diet interventions. To get a brief impression of Plin expression, liver extract from 89 mice was prepared, and samples from each treatment group were subsequently pooled into one sample and loaded in duplicate on the SDS-PAGE gel. A) Plin2 immunoblot signal. B) Plin3 immunoblot signal. Protein expression was normalized to total protein measured by staining with Ponceau S, and the expression in the chow group. Abbreviations used: C: control group; D: diet group; chow (n=9); fast (n=10); LFS: low fat-cholesterol-sucrose diet (n=8); HFS: high fat-cholesterol-sucrose diet (n=8); LF: low fat diet (n=10); HF: high fat diet (n=12); LFK: low fat ketogenic control diet (n=8); HFK: high fat ketogenic diet (n=8); MCDC: methionine and choline deficient control diet (n=8); MCD: methionine and choline deficient diet (n=8).

5.3 Liver gene expression

5.3.1 Housekeeping genes

To be able to investigate gene expression in liver, several genes believed not to be affected by the diet interventions were tested to find an appropriate reference gene (figure 5.4). Special care was taken to include the same amount of cDNA for all samples. Hence, no difference in expression is expected for an unregulated gene. Expression of the gene *Tbp* was significantly affected by fasting and the MCD diet, while expression of the gene *Rplp0* was significantly affected by HF, HFK and the MCD diets. The gene *Ppib* was significantly affected by fasting. The genes *Polr2a* and *Rpl32* were unaffected by all treatments and determined to be appropriate reference genes for these experiments. Based on these analyses, *Rpl32* was considered to be the most stable expressed gene and was selected as reference gene for all hepatic gene expression analysis in this master thesis.

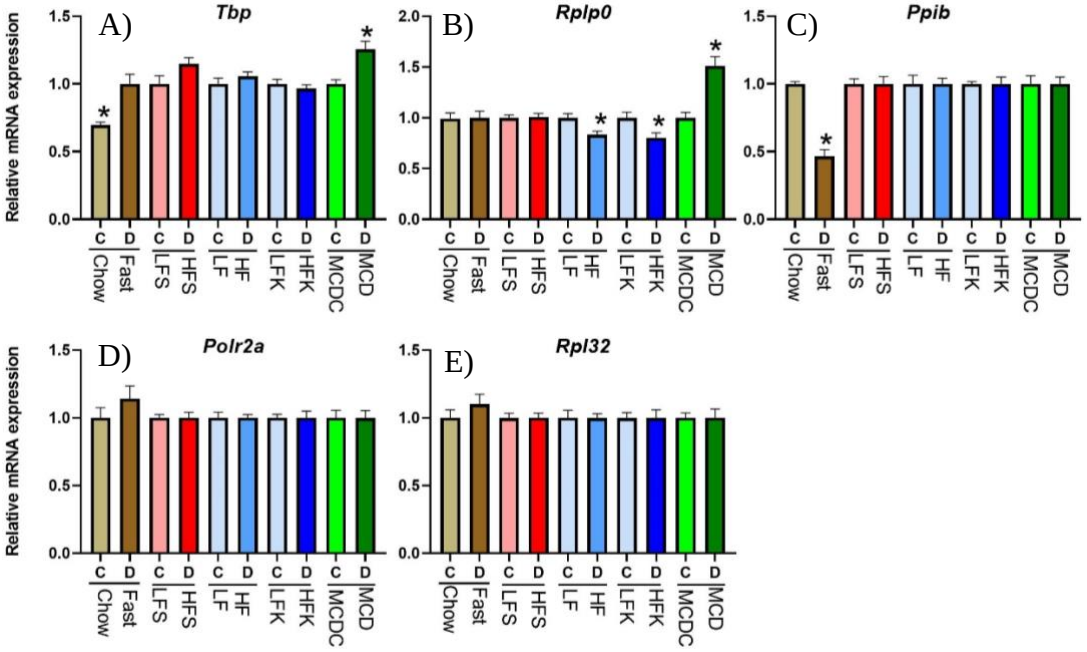

Figure 5.4. Hepatic mRNA expression of housekeeping genes. Relative mRNA expression of selected genes to test their robustness as reference gene for liver samples of fasted mice or mice receiving various diets. A) *Tbp*, B) *Rplp0*, C) *Ppib*, D) *Polr2a*, E) *Rpl32*. Expression values are expressed as $2^{-\Delta Ct}$, relative to the expression of Rpl32. Abbreviations used: C: control group; D: diet group; chow (n=9); fast (n=10); LFS: low fat-cholesterol-sucrose diet (n=8); HFS: high fat-cholesterol-sucrose diet (n=8); LF: low fat diet (n=10); HF: high fat diet (n=12); LFK: low fat ketogenic control diet (n=8); HFK: high fat ketogenic diet (n=8); MCDC: methionine and choline deficient control diet (n=8); MCD: methionine and choline deficient diet (n=8). Significant differences between the control group and the intervention group are marked with stars (p-value <0.05). Bars are mean + SEM.

5.3.2 Genes related to lipid droplet binding

Storage of TAG in LDs depends on a balanced expression of LD surface proteins. Expression of genes belonging to the LD-binding *Plin* and *Cide* families was therefore analyzed. Fasting and all the different diet exposures resulted in a general increase in the expression of one or more of the LD binding genes, with a few exceptions (figure 5.5). Of the five plins, *Plin2* had the highest relative mRNA expression, followed by comparable levels of *Plin3*, *Plin4* and *Plin5*, while *Plin1* seemed less expressed. *Cideb* had the highest expression among the *Cide* genes, followed by *Cidec* and even lower expression levels of *Cidea*. Fasting resulted in a particularly strong upregulation of *Plin2*, *Cidec*, *Plin4* and *Plin5* expression. Most diets resulted in unchanged or a minor increase in the expression of *Plin* and *Cide* genes, with a few exceptions. MCD diet gave a strong increase in the expression of *Plin4* and *Cidec* and these inductions stood out compared to the other diets. The HFS diet increased expression of a higher number of LD-genes compared to the other diets. *Plin2* and *Plin5* expression were essentially similarly upregulated by fasting and all diet interventions (except for the MCD diet), suggesting that transcription of these two genes are similarity regulated. In conclusion, several of the LD binding proteins were upregulated in the dietary interventions, but particularly *Plin4* and *Cidec* were substantially upregulated by the HFS or MCD diets.

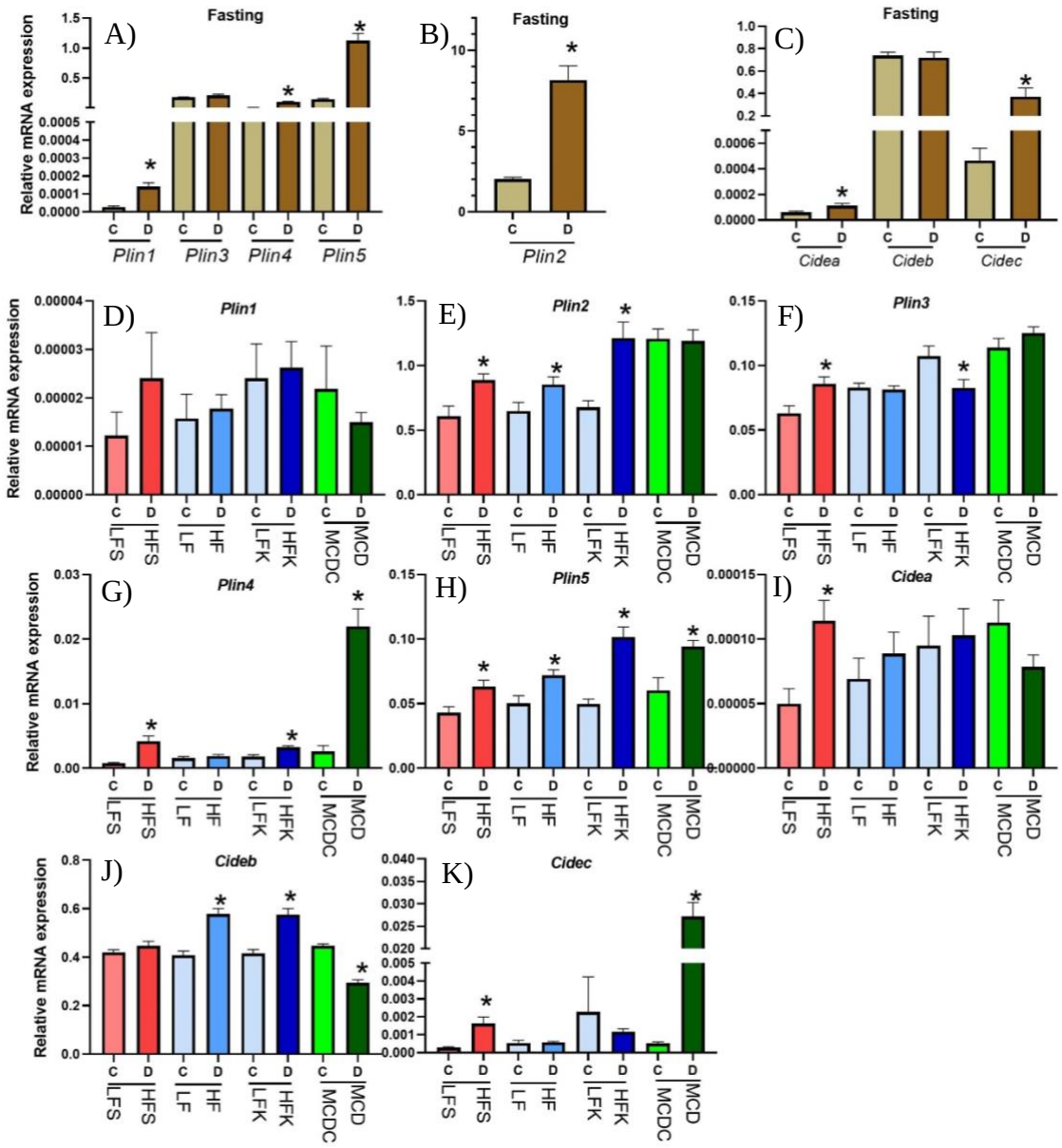

Figure 5.5. Hepatic mRNA expression of genes related to LD binding. A-C) Relative mRNA expression of *Plin* and *Cide* LD-binding genes in chow fed and fasted mice, D-K) Relative mRNA expression of *Plin* and *Cide* LD-binding genes among the mice from the different diet intervention studies. Expression values are expressed as $2^{-\Delta Ct}$, relative to the expression of *Rpl32*. Abbreviations used: C: control group; D: diet group; chow (n=9); fast (n=10); LFS: low fat-cholesterol-sucrose diet (n=8); HFS: high fat-cholesterol-sucrose diet (n=8); LF: low fat diet (n=10); HF: high fat diet (n=12); LFK: low fat ketogenic control diet (n=8); HFK: high fat ketogenic diet (n=8); MCDC: methionine and choline deficient control diet (n=8); MCD: methionine and choline deficient diet (n=8). Significant differences between the control group and the intervention group are marked with stars (p-value <0.05). Bars indicate mean + SEM.

5.3.3 Genes related to lipolysis and FA oxidation

Lipolysis is the process of hydrolyzing TAGs into glycerol and FFAs, and is usually upregulated when energy expenditure is higher than the caloric intake. Several genes central

to fatty acid oxidation are regulated by transcription factors, such as *Pparα*, which is activated by ligand binding to FAs (85). The intake of FAs, in addition to the quantity and type of fats ingested from the diet may therefore play a role in the expression of genes related to lipolysis and fatty acid oxidation, and was therefore measured. Fasting and the diets high in fat resulted in increased expression of some, but not all of the lipolytic genes (figure 5.6). The *G0s2* mRNA was increased in fasted mice and mice fed the HFS or HFK diets, whereas the *Pnpla2* mRNA was increased in all intervention groups except for in mice receiving a HFS diet. The *Abhd5* and *Lipe* mRNAs were unaltered in their expression. Two genes involved in oxidation of FAs were also measured. The *Acox1* and *Cpt1a* mRNAs were induced in expression by fasting and diets with high lipid content (HFS, HF, HFK diets.). Expression of *Acox1* and *Cpt1α* were similarly upregulated by fasting and all diets high in lipids, suggesting that the transcription of these two genes are similarly regulated.

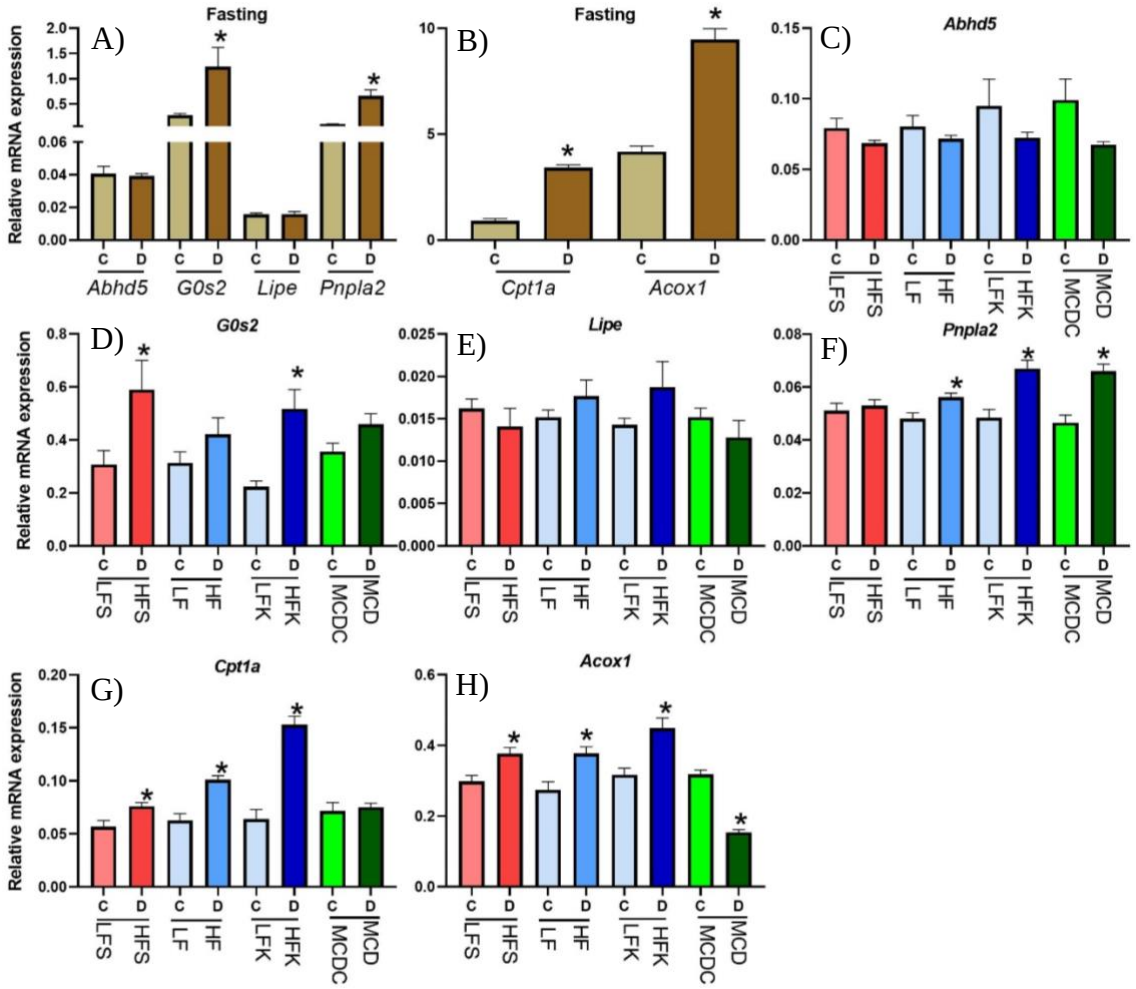

Figure 5.6. Hepatic mRNA expression of genes related to lipolysis and lipid oxidation. A)-B); Expression of lipolytic and fatty acid oxidizing genes among fasting mice, C)-H); Expression of lipolytic and fatty acid oxidizing genes among the mice from the different diet intervention studies. Expression values are expressed as $2^{-\Delta Ct}$, relative to the expression of *Rpl32*. Abbreviations used: C: control group; D: diet group; chow (n=9); fast (n=10); LFS: low fat-cholesterol-sucrose diet (n=8); HFS: high fat-cholesterol-sucrose diet (n=8); LF: low fat diet (n=10); HF: high fat diet (n=12); LFK: low fat ketogenic control diet (n=8); HFK: high fat ketogenic diet (n=8); MCDC: methionine and choline deficient control diet (n=8); MCD: methionine and choline deficient diet (n=8). Significant differences between the control group and the intervention group are marked with stars (p-value <0.05). Bars are mean + SEM.

5.3.4 Genes related to lipogenesis and TAG packaging

Lipogenesis, the synthesis of FAs from acyl-CoA, and incorporation of these into TAG is highly affected by the diet (114). A diet high in carbohydrates is expected to stimulate lipogenesis, whereas a diet high in lipids is expected to repress lipogenesis. Transcription factors such as *Lxr-*, *Chrebp-* and *Srebf1* isoforms are central for lipogenesis in liver, and was therefore analyzed together with selected downstream genes. Fasting gave a general decrease in genes related to lipogenesis, including *Srebf1c* and *Chrebp*s transcription factors, FA

synthesis genes (*Fasn* and *Acc*), the desaturase *Scd1* and the FA esterification genes *Dgat1* and *Dgat2* (figure 5.7). The HFS diet increased expression of a larger number of lipogenetic genes compared to the other diets, and all the transcription factors measured had a tendency of being upregulated. The HF and the HFK diets resulted in an upregulation of the *Lxr*s and esterification gene *Dgat1*, while the remaining transcription factors (*Srebf1* and *Chrebp*s) measured and several downstream genes (*Fasn, Acc* and *Scd1*) were downregulated, indicating a reduced synthesis of FAs (figure 5.8). The MCD diet showed some similarities in gene expression to the fasting mice, with a reduction in the transcription factors *Srebf1c* and *Chrebp*s, and downstream genes such as *Fasn, Acc* and *Scd1* compared to their controls. The gene *Scd1* had the highest expression in liver among all the diets, and had a noticeable high expression among the mice on the HFS diet, while it was downregulated and less expressed among the remaining diets. In conclusion, diets low on carbohydrates (HF and HFK) had a robust reduction in the expression of transcription factors related to lipogenesis (*Chrebp*s and *Srebf1c*), in addition to downstream genes (such as *Fasn, PkLr* and *Acc*), indicating downregulation of lipogenesis on such a diet.

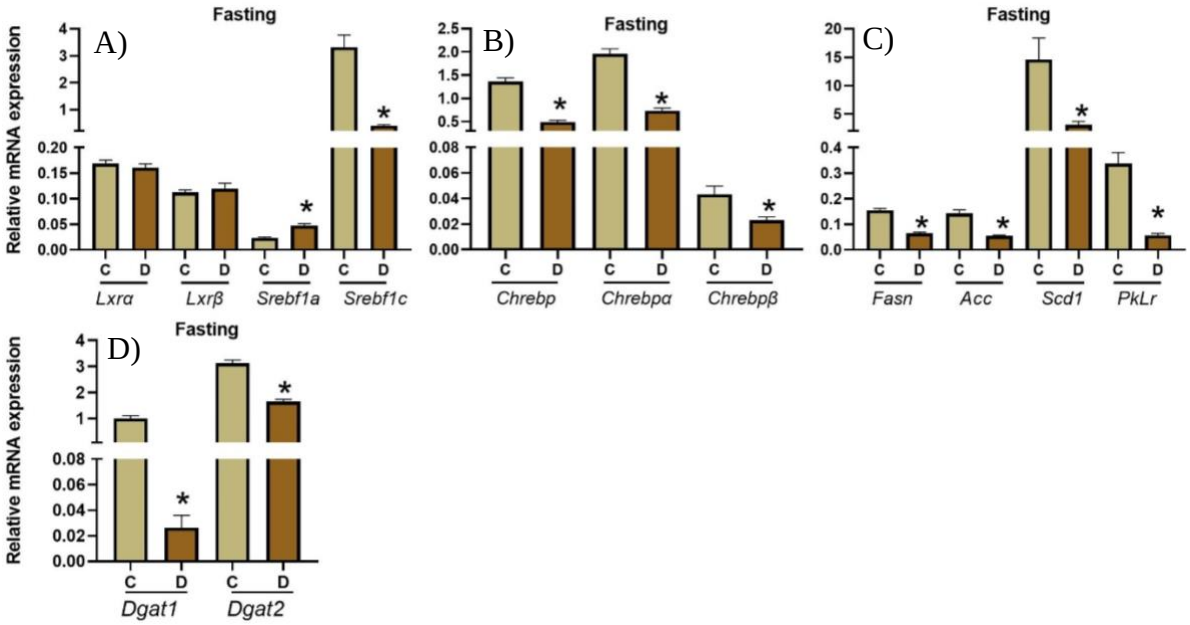

Figure 5.7. Hepatic mRNA expression of genes related to lipogenesis and TAG packaging among fasted mice. A); Expression of transcription factors related to lipogenesis and TAG packaging, B); Expression of transcription factors related to glucose and lipid metabolism, C); Expression of genes related to synthesis of TAG and oxidation of glucose (PkLr) and lipid metabolism, D); Expression of genes related to TAG production. Expression values are expressed as $2^{-\Delta Ct}$, relative to the expression of the housekeeping gene. Abbreviations used: C: control group; D: diet group; chow (n=9); fast (n=10). Significant differences between the control group and the intervention group are marked with stars (p-value <0.05). Bars are mean + SEM.

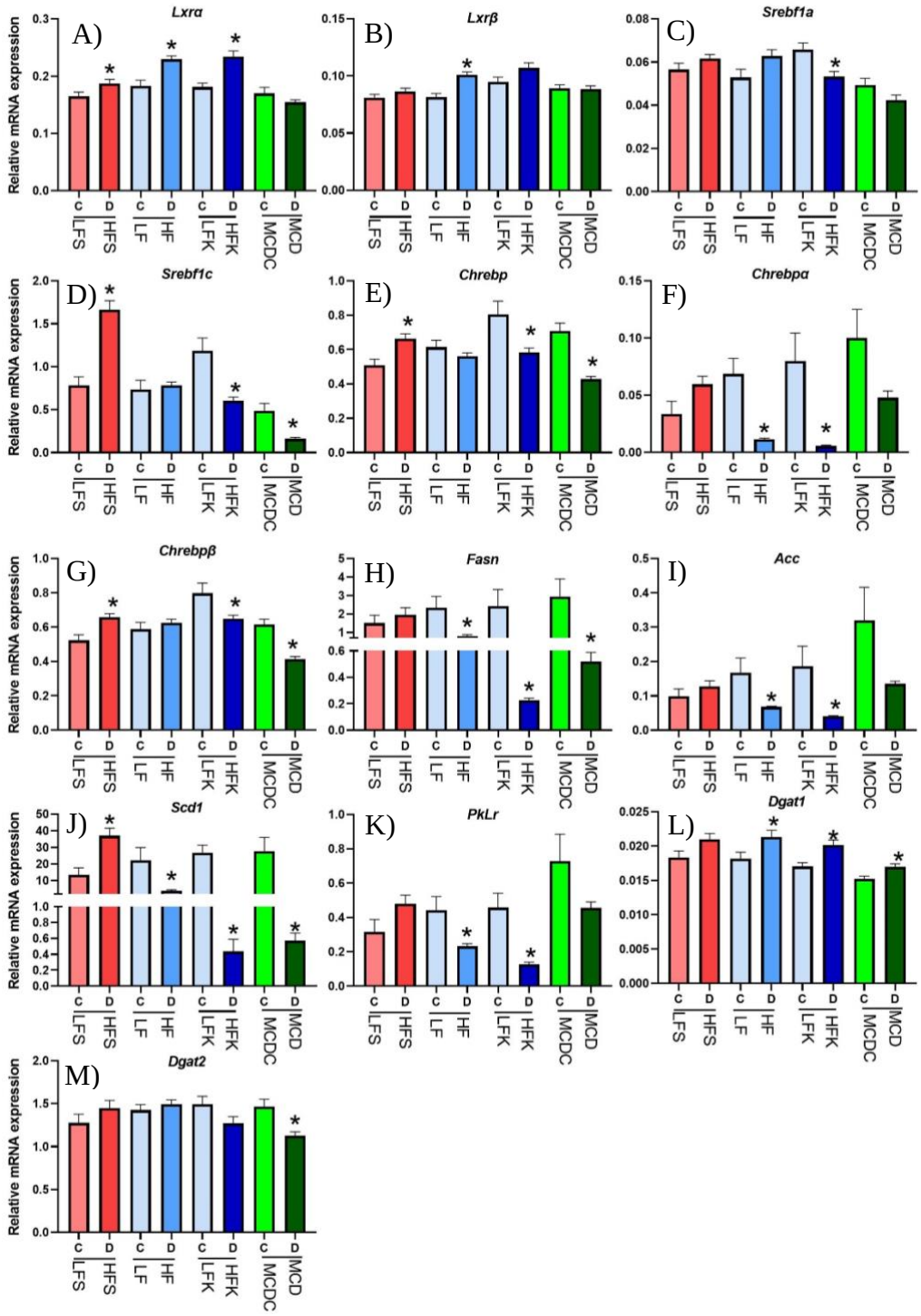

Figure 5.8. Hepatic mRNA expression of genes related to lipogenesis and TAG packaging among mice on the remaining diet intervention studies. A-G); Expression of transcription factors related to lipogenesis and TAG packaging, H-M); Expression of genes related to synthesis of TAG and oxidation of glucose (PkLr). Expression values are expressed as $2^{-\Delta Ct}$, relative to the housekeeping gene. Abbreviations used: C: control group; D: diet group; chow (n=9); fast (n=10); LFS: low fat-cholesterol-sucrose diet (n=8); HFS: high fat-cholesterol-sucrose diet (n=8); LF: low fat diet (n=10); HF: high fat diet (n=12); LFK: low fat ketogenic control diet (n=8); HFK: high fat ketogenic diet (n=8); MCDC: methionine and choline deficient control diet (n=8); MCD: methionine and choline deficient diet (n=8). Significant differences between the control group and the intervention group are marked with stars (p-value <0.05). Bars represent mean + SEM.

5.3.5 Expression of fatty acid responding transcription factors

The transcription factors *Ppar*s are activated by FAs and was therefore analyzed (115). The *Ppar*s had a relatively low expression in liver, with *Pparα* having the highest expression, followed by *Pparδ* (figure 5.9). *Pparα* mRNA was upregulated by fasting and all diets high in lipids (HFS, HF, HFK), while *Pparδ* mRNA was downregulated by fasting and most of the same diets compared to the respected controls. The Pparγ mRNA was higher expressed by most treatment diets. The *Ppar* co-activators *Pgc1a* and *Pgc1b* were increased in expression by fasting and to some extent by diets high in lipids. Interestingly, the MCD diet decreased the expression of *Pparα*, despite a high level of liver TAG accumulation in these animals.

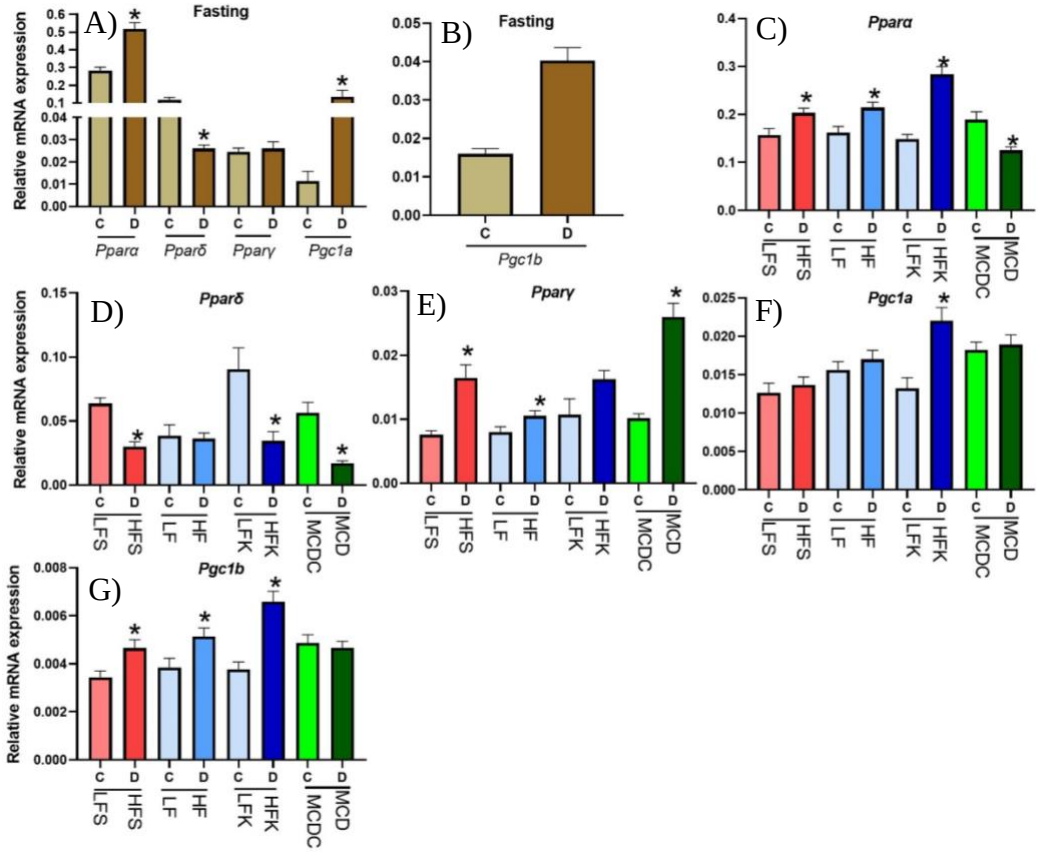

Figure 5.9. Hepatic mRNA expression of transcription factors related to the metabolism of FAs. A)-B); Expression of *PPARs* and their co-activators among fasting and chow fed mice, C)-E); Expression of *PPARs* among mice on the remaining diet intervention studies, F)-G); Expression of co-activators of the transcription factors *PPARs* among mice on the remaining diet intervention studies. Expression values are expressed as $2^{-\Delta Ct}$, relative to the expression of *Rpl32*. Abbreviations used: C: control group; D: diet group; chow (n=9); fast (n=10); LFS: low fat-cholesterol-sucrose diet (n=8); HFS: high fat-cholesterol-sucrose diet (n=8); LF: low fat diet (n=10); HF: high fat diet (n=12); LFK: low fat ketogenic control diet (n=8); HFK: high fat ketogenic diet (n=8); MCDC: methionine and choline deficient control diet (n=8); MCD: methionine and choline deficient diet (n=8). Significant differences between the control group and the intervention group are marked with stars (p-value <0.05). Bars are mean + SEM.

5.3.6 Expression of inflammation markers

A diet lacking methionine and choline is often used as a dietary model to induce steatosis and inflammation in the liver, and a few selected inflammation markers were therefore analyzed. Fasting gave a significant decrease in the expression of *Tnf* while it tended to increase *Il1b*, even though both had a low expression in liver (figure 5.10). The MCD fed mice resulted in a higher expression of the inflammation markers, which are expected on such a diet. The diets low in carbohydrates (HF and HFK) had a lower expression of *Il1b*, while it was higher expressed in the diets rich in carbohydrates (LF, HFS and LFK) and the diet lacking the essential amino acids methionine and choline.

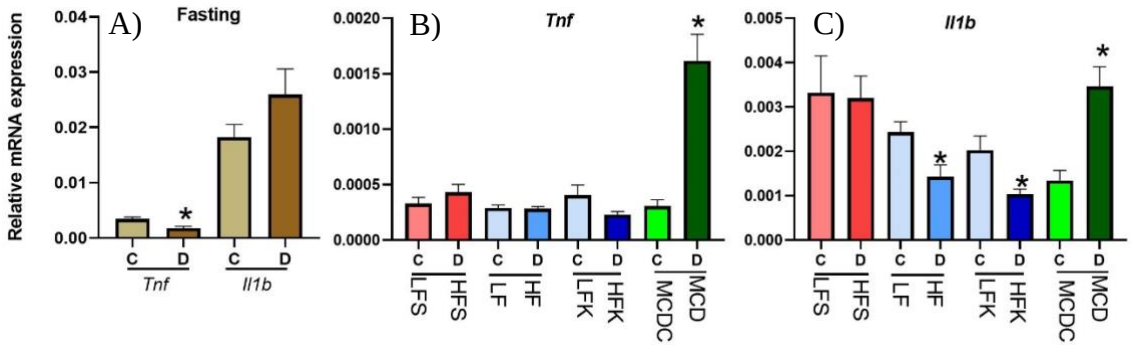

Figure 5.10 Hepatic mRNA expression of inflammation markers. A): Expression among fasting mice, B)-C); expression among mice on the remaining diet intervention studies. Expression values are expressed as $2^{-\Delta Ct}$, relative to the control group for each diet intervention study. Abbreviations used: C: control group; D: diet group; chow (n=9); fast (n=10); LFS: low fat-cholesterol-sucrose diet (n=8); HFS: high fat-cholesterol-sucrose diet (n=8); LF: low fat diet (n=10); HF: high fat diet (n=12); LFK: low fat ketogenic control diet (n=8); HFK: high fat ketogenic diet (n=8); MCDC: methionine and choline deficient control diet (n=8); MCD: methionine and choline deficient diet (n=8). Significant differences between the control group and the intervention group are marked with stars (p-value <0.05). Bars are mean + SEM.

Table 5.1. Summary of the different diet intervention studies included and their impact on metabolic parameters measured. This table summarizes how the different diet intervention studies influenced parameters such as bodyweight and body composition, in addition to hepatic gene expression of genes related to LD binding, lipogenesis, lipid oxidation and inflammation. Green arrows indicate gain of bodyweight, fat mass and liver mass or an upregulation in gene expression, whereas red arrow indicates a loss or downregulation. Abbreviations used: HFS: high fat-cholesterol-sucrose diet (n=8); HF: high fat diet (n=12); HFK: high fat ketogenic diet (n=8); MCD: methionine and choline deficient diet (n=8).

	Diet intervention				
Parameters measured	**Fasted**	**HFS**	**HF**	**HFK**	**MCD**
Bodyweight	↓	↑	↔	↔	↓
Fat mass	↓	↑	↑	↔	↓
Liver fat	↑	↑	↔	↔	↑
LD binding genes	↑	↑	↑	↑	↑
Lipogenesis (*Srebf1c, Fasn, Acc*)	↓↓	→↑	↓↓	↓↓	↓
Lipid oxidation (*Cpt1, Acox1*)	↑	↑	↑	↑	→↓
Ppar alpha	↑	↑	↑	↑	↓
Inflammation	(↓)	↔	(↓)	(↓)	↑↑

6 Discussion

6.1 Animal models

6.1.1 Mouse models for studying metabolic syndrome

Animals are widely used as preclinical models to understand human biology, by studying various molecular and physiological mechanisms believed conserved to humans. Rodents, especially mice, have been crucial in biomedical research to understand overconsumption of calorie rich food and pathologic effects of obesity. Mice are readily available, easy to maintain at low costs, which makes them a great model organism. In addition, mice can be made genetically identical (inbreed strains), which makes it easier to identify differences between groups with relatively few individuals with higher reproducibility between different studies. Although, mice and humans share similarities in organ systems, systemic physiology, energy metabolism and disease pathogenesis, it is important to remember that they are two different species with distinct metabolic rate, giving them significantly differences regarding life expectancy and fertility, physiology and morphometry (116). Despite clear species differences between humans and animals, animal studies using rodents are the most important and valuable research tool available to increase our understanding of human biology and disease, and well suited to investigate complex pathology as a result of caloric overconsumption and obesity (117).

The C57BL/6N mice is often used as a model for studying weight gain and metabolic syndrome with glucose intolerance as a result of caloric overconsumption, especially on dietary fat (118, 119). The C57BL/6N strain has a relatively stable weight gain until 6-7 weeks of age on a regular diet before the weight stabilizes (120). Two of the diet intervention studies were done on the mice from the age of 8 weeks and until the age of 20 weeks, which is due to the stabilization of the weight gain around 7 weeks. Female C57BL/6N mice have a normal body weight of around 24 grams at 20 weeks of age (121), and a higher body weight can be concluded to represent obesity. This strains is known to be prone to diet-induced obesity (DIO) and is considered to be a good model for metabolic syndrome affecting obese humans, due to its similarities in the development of obesity and obesity-related

complications when eating a high-fat/high-density “Western diet” (122). The DIO model usually better mimics the dietary causes of human development of obesity, while a knockout model is better suited for studying a particular gene or pathway in the development of obesity (123). A DIO mouse model was considered the better option for this master thesis and were used as a representative model for human metabolic syndrome (124). The metabolic effects of mice with obesity, such as changes in bodyweight, fat mass, liver mass and accumulation of liver lipids show many similarities with obese humans eating a high-energy diet. The results from the mice may therefore give an insight into the pathophysiology and development of obesity and its related complications (125).

Different diets and development of NAFLD and NASH

The used MCD diet is deficient in the essential nutrients methionine and choline, and this diet is not regularly consumed by humans. The diet induces weight loss rather than weight gain, which is contradictory since obesity is a predisposing factor for metabolic syndrome and risk of developing NAFLD. Despite this, the MCD diet is widely used as a model for liver inflammation and investigation of metabolic changes leading to the development of NASH, since the MCD diet leads to a similar inflammation in the liver and eventually NASH (126). The MCD diet is a fast approach to investigate mechanisms that cause NAFLD to develop into advanced NASH and fibrosis, while the HFS, often called the “Western diet”, better mimics human NAFLD (124).

6.1.2 The use of female mice

In this master thesis, female mice, rather than male mice, from the strain C57BL/6N was used. There is a lack of using female mice models in medical and nutritional research, which may lead to poorer treatment options for women compared to men (127). The general use of male mice instead of female mice in animal experiments, is due to a general belief that there is higher variability in both gene expression levels and hormone levels in female mice, in addition to variations caused by the estrous cycle. In contradictory to beliefs, male mice have shown a slightly higher, significant variability compared to female mice, and the estrous cyclicity did not seem to be a major source of variability (127). Based on all current

knowledge, female mice was deemed to be an appropriate model for this master thesis, and was used since studies in female mice are underrepresented in the available literature.

6.1.3 Limitations with dietary intervention studies

The biological samples analyzed in this project originated from five independent diet intervention studies performed at different time points, which may lead to systemic differences between the studies. If a similar project is going to take place in the future, it will be favorable to do all diets within the same time-frame to enable direct comparison between all diets, although this is certainly very difficult and time-demanding to arrange. The age of the mice used varied somewhat between the diet intervention studies, but mice were matched in age between the control group and the intervention group for each diet intervention. A difference in age will influence bodyweight, body composition and organ weight, but this was not considered a major problem in this study since no direct comparisons were conducted between the different diet intervention studies. The mouse strain C57BL/6N is widely used in research related to DIO as they develop obesity with prolonged intake of a high fat diet (118, 119). In our study, mice receiving the HF diet were significantly leaner than expected when compared to comparable diet interventions (128, 129). This could be due to several reasons. If the high fat diet is left for too long in the home cage it becomes stale, which may lead to reduced food intake. Some mice were placed in metabolic cages during the diet intervention, which leads some mice to thrive less and eat less (130). The small weight gain in the HF group may therefore have been caused by methodological bias. Future investigations should use mice from a better controlled HF study to compare differences in body composition, gene and protein expression compared against the other diets. The mice fed the HFK diet were only exposed to the diet for four weeks, while the HFS and the HF diet were conducted for 12 weeks. It is not clear whether four weeks is long enough to give significant changes in all parameters measured, or if it requires a longer intervention to yield significant results.

6.2 Methods used

6.2.1 RT-qPCR

To investigate the relative mRNA expression of genes related to LD binding, lipolysis, lipid oxidation, lipogenesis, lipid esterification, transcription factors and inflammation markers, liver tissue from the mice was sampled, the RNA isolated and converted to cDNA, and expression levels of individual genes analysed with qPCR. Tissue from the same liver segments was tried sampled, but proved difficult due to differences in liver size. Variations in the tissue segment used may lead to some differences in the relative gene expression and influence the results, since different liver segments have slightly different gene expressions, called metabolic zonation (131).

The isolated RNA had a high purity with a concentration sufficient for downstream analyses. RT-qPCR is an efficient and cost-efficient method to analyse gene expression, and when optimal and specific primers are designed, the method has a high specificity and sensitivity (132). In this master thesis, the dye SYBR Green was chosen to detect the amplicons from the RT-qPCR, as it is a less expensive dye compared to other alternatives. The limitations of choosing a dye-based method instead of a probe-based one is that the dye can bind to the target sequence and any unspecific amplified sequences in the same sample. It is important to choose a good primer which binds the gene of interest to avoid detection of unspecific DNA in the samples, since SYBR Green will bind to any double stranded DNA available in the samples. Gene expression was calculated as $2^{-\Delta Ct}$, relative to the housekeeping gene *Rpl32*. This method assumes that the target sequence is doubled in each RT-qPCR cycle. To confirm that the reaction reaches the required efficiency, a cDNA dilution curve should be run alongside the samples, but this is very time consuming and hard to do in the time frame given for this master thesis, in addition to everything else that needed to be done. With a probe-based qPCR, the chance of binding unspecific DNA is smaller, but this requires well-designed specific probes, that are expensive. A melt curve analysis was run for all the RT-qPCRs to confirm the absence of multiple amplicons or primer dimers, in addition to including negative controls in the RT-qPCR analyses. The primers used in this master thesis and the amplification of the target sequences was therefore assumed specific and the results applicable.

The study consisted of samples from 10 different groups receiving diets with different composition, and identification of an appropriate reference gene was important to be able to normalize gene expression data. Most of the reference genes tested were influenced by one or more of the diets and could not be used. After some testing, the gene *Rpl32* was deemed to be the most stable expressed gene. Five independent diet intervention studies were included in this master thesis, where the five control groups received diets with relatively similar nutritional composition (see table 4.5). Gene expression levels for the majority of genes measured in the control groups seemed to be quite comparable, which suggests that there is a relatively high reproducibility in the measured gene expression data between repeated dietary interventions.

6.2.2 Measuring the hepatic content of fat and Plin protein expression

Measuring the hepatic content of lipids

To be able to measure the absorbance of the lipid samples, the samples had to be diluted 32 times to fall within the linear standard curve. Samples with a low absorbance, which results in less signal, may result in less precise measurements, since the signal is easily disturbed by contamination or cellular debris in the samples, blocking the light and giving a false high reading. The absorbance of all samples fell within the standard curve and was deemed sufficient for further use. A kit made for measuring cholesterol esters was tested, but the results were inconclusive and was deemed inappropriate for use. The low levels of liver cholesterol renders it necessary to perform Folch's extraction to consecrate the lipids prior to measurement of cholesterol. This procedure is very time-consuming and difficult to implement on the large number of samples included in this study, and was therefore not conducted.

Western blotting

Western blot was ongoing when the outbreak of the COVID-19 happened and hindered further work in the laboratory. Individual samples of each group were pooled before they were loaded on the gel. This strategy was used to get a first impression of the Plin protein

content before selecting certain diets for western analysis where individual samples were loaded. The membranes were stained with Ponceau S to measure the total protein in each lane. This revealed some variation in protein abundance, which may have increased the variability. The band intensities were manually measured in ImageJ by selecting multiple peaks and subtracting the background intensity, as previously described (133). This method has its limitations, and can lead to variations based on the individual assessing the results (134). Despite this, the method was considered satisfactory for a screening purpose and was used to quantify signals on both western blots.

6.2.3 Missing analysis

In this master thesis, neither the cholesterol content in the liver, nor the glucose tolerance have been tested. Both these parameters can be used to assess metabolic health and metabolic syndrome among the mice, in addition to its transferability to human metabolic syndrome. These methods were deemed too time-consuming and expensive for this established project, and it was therefore determined to focus on measurement of TAG and other methods instead, such as RT-qPCRs and western blots.

6.3 Results from the diet interventions

The different diet intervention studies led to several distinct changes in the metabolic factors measured. To summarize the key findings, both fasting and MCD fed mice lost a substantial amount of bodyweight and fat mass, but had increased hepatic lipid accumulation and protein expression of Plin2. The MCD fed mice also showed a high expression of LD binding genes *Plin4* and *Cidec*, which has never been observed before. The mice fed the HFS diet, which is rich in saturated fat, cholesterol and sucrose gained both bodyweight and fat mass, and had a high accumulation of TAGs in liver and hepatic protein expression of Plin2. The HFS fed mice also showed increased expression of lipogenic genes and central transcription factors, indicating enhanced FA synthesis in these mice. The diets rich in fats and low in carbohydrates showed no significant changes in bodyweight or fat mass, nor any hepatic lipid accumulation despite a higher expression in Plin2 and Plin3 protein. The HF and HFK fed mice had a tendency of upregulating LD binding and lipolytic genes, indicating a seemingly higher oxidation and clearance of lipids in liver.

6.3.1 The MCD diet

The MCD diet is often used as a model for NASH and liver fibrosis, where the lack of methionine and choline induces hepatic lipid accumulation and inflammation (124). Weight gain is considered to be an important contributing factor to the development of NAFLD in humans (26, 33, 135). Still, the MCD diet was the only diet that led to a decrease in both bodyweight and fat mass compared to animals eating the control diet, which is thought to be due to increased metabolic activity (124, 136, 137). The MCD fed mice also had the highest liver weight relative to bodyweight, and displayed a substantial upregulation in the relative protein expression of Plin2 in the liver in the western blot, in addition to a higher accumulation of TAGs, which could imply the presence of NAFLD (138-140). As mentioned, *Pparα* is central for regulating genes related to lipid oxidation, such as *Cpt1α* and *Acox1* (84, 85). Interestingly, both *Pparα* and *Acox1* were significantly downregulated on the MCD diet, which may indicate that the FA oxidation was not increased, despite high FA accumulation in this mouse model (141). Methionine and choline have been shown to be important for VLDL secretion in liver, since these amino acids are the precursor to phatidylcholine, which coats the VLDL particles (141). A lack of these essential amino acids leads to impaired hepatic VLDL secretion, which is theorized to lead to hepatic steatosis (141, 142). Both the transcription factors *Srebf1c* and *Chrebp* were downregulated on the MCD diet, in addition to downstream genes such as *Fasn*, *Acc* and *Scd1*, indicating a decrease in the hepatic lipid synthesis, which may have an influence on the lipid loading of VLDL particles (141).

Despite the mice developing NAFLD on the MCD diet, the pathology of this is not identical to the human development of NAFLD, raising the question whether the MCD diet is a representable model for human NAFLD, or if it is best suited as a model for NASH (124, 137). This diet also resulted in an especially high increase in gene expression of the LD binding genes *Plin4* and *Cidec*, which has never been reported previously. The MCD diet was also the only diet which led to a significant increase in hepatic gene expression of the inflammation markers *Il1b* and *Tnf,* indicating the presence of inflammation (124). Since no histology for fibrosis were conducted, it is not possible to draw any conclusions regarding the degree of fibrosis. In this master thesis, only mRNA expression was measured, which cannot fully describe enzyme or metabolic activity, and no definitive conclusion can be drawn regarding these parameters.

6.3.2 Fasting

Mice deprived of food for 24 hours showed a significant decrease in bodyweight, liver weight and fat mass compared to their controls. The fasting mice showed a significant increase in the hepatic lipid accumulation, in addition to a higher expression of numerous LD binding genes, and especially a large increase in the expression of *Plin2*, which is strongly connected to LD binding in liver. In addition, the fasting mice had a high increase in the expression of *Cidea* and *Cidec*, which is transcriptionally regulated by *Pparα* and hypothesized to be responsible for hepatic lipid accumulation under both obese and fasted conditions (143, 144). Both *Cidea* and *Cidec* are primarily found in adipose tissue, while *Cideb* is the primarily CIDE expressed in liver (143, 145). *Pparα* is central in lipid oxidation, and was upregulated in fasting mice. The same was seen for the downstream LD binding proteins *Plin2* and *Plin5*, and the enzymes *Cpt1α* and *Acox1*, which are central in FA oxidation, indicating a higher oxidation of FAs (144). Mice fed a low carbohydrate diet had a low expression of glucose responsive transcription factors (*Srebf1c* and *Chrebp*) and lipogenetic genes (*Fasn, Acc, Scd1* and both *Dgat*s), indicating downregulation of FA synthesis (49). During fasting, the adipose tissue will hydrolyze TAG, releasing FFAs as substrates to the general circulation for energy production. The release of FFAs from the adipose tissue will lead to a high influx of FA to the liver, which explain the observed results with increased lipid accumulation, lipid oxidation and decreased synthesis of lipids (49).

Similarities between fasting and the MCD diet

The high similarity between fasted mice and the MCD fed mice is interesting. These mice shared a loss in bodyweight and fat mass, a higher accumulation of TAGs and expression of Plin2, which is strongly connected to LD binding in liver, suggesting a similar regulation of Plin2 and accumulation of hepatic LDs. Hepatic lipid accumulation is physiological response to fasting, or a consequence of a disturbances in the homeostasis of hepatocellular synthesis and removal of TAGs, which includes numerous pathways (rate of FA uptake, the esterification and lipolysis of FAs, rate of lipid oxidation and synthesis), and several of these mechanisms could be responsible for hepatic lipid accumulation (142). While the hepatic expression of lipolytic genes, such as *Pparα*, *Cpt1α* and *Acox1* was upregulated among the fasting mice, the same genes showed a downregulation among the MCD fed mice, indicating

a difference in the regulation of oxidation of lipids. Both diets also showed a significant upregulation of the gene *Cidec*, which has been shown to be regulated by *Pparγ* (146). The expression of *Pparγ* was upregulated with the MCD diet, while the other *Ppars* were downregulated compared to the control group. While both diets led to accumulation of hepatic lipids and upregulation in central LD binding genes, only the MCD diet showed signs of inflammation and increased liver mass, which is in accordance with development of steatohepatitis (26, 124). Importantly, the MCD diet is a severe and strict diet insufficient in several essential nutrients and is not representable for any human diet.

6.3.3 High fat high sucrose diet

Mice fed a diet high in sucrose, fat and cholesterol, often termed as "the Western diet", had increased bodyweight, fat mass, liver weight and accumulation of liver TAGs compared to mice fed the control diet. Such a diet is often connected to obesity, metabolic syndrome and NAFLD among humans, showing similarities between the two species regarding diet exposures (124, 147, 148). The increase in liver weight may partly be a result of increased liver TAGs, which is supported by an increase in the expression of Plin2 shown in the western blot and the RT-qPCR, as Plin2 is important for accumulation and formation of LDs in liver (86, 90, 113, 149). The high content of cholesterol and sucrose in a high fat diet has been shown to be central in the accumulation of lipids in the liver and disturbances in the lipid metabolism (150-154). The increased liver fat was accompanied by an increase in several genes related to LD binding and lipogenesis, and a slight increase in genes involved in β-oxidation, such as *Pparα, Acox1* and *Cpt1α*, which has been observed earlier in similar diet intervention studies (126). The transcription factors *Lxrα, Srebf1c* and *Chrebp*, which are central in the lipogenesis, were upregulated, but *Scd1* was the only downstream lipogenetic gene which showed any significant upregulation on this diet. *Pparα* has also been shown to influence lipogenesis through regulation of the transcription factor *Lxrα* and subsequent increases in the transcription of *Scd1*, and may play a role in lipogenesis through these mechanisms (155, 156). Despite not observing any other significant differences, the remaining genes measured related to lipid synthesis showed tendencies of being upregulated, indicating increased lipogenesis. This can be hypothesized to be a result of the high content of fat and cholesterol in the diet, in addition to the high content of sucrose (157). Such a diet has

been shown to be especially unhealthy, resulting in weight gain and accumulation of fat in the liver and increased risk for development of metabolic syndrome, inflammation and dysbiosis in the gut microbiome (124, 152, 158, 159).

It is hypothesized that the high content of added sugars, such as sucrose and high fructose corn syrup (HFCS), in the typical Western diet promotes the development of NAFLD (160), type II diabetes and cardiovascular disease through both direct and indirect mechanisms (161). Ingested fructose is transported into liver cells via GLUT2, where fructose is converted to fructose-1-phosphate by the enzyme fructokinase, which, in contrast to glucokinase is not regulated by the hepatic energy status (160-162). Further conversion of fructose leads to excess acetyl-CoA, which further promotes hepatic *de novo* lipogenesis and production of FAs, while suppressing transport of FAs by *Cpt1α* into the mitochondria and oxidation of FAs. The increase in hepatic lipids induces the production and secretion of VLDLs, which further increases the amount of circulating TAGs and LDLs in the bloodstream and the risk of developing CVD (161). A high, chronic intake of fructose activates transcription factors such as *Chrebp* and *Srebp1c*, which is important for the regulation and expression of enzymes central for the lipid metabolism (160). In addition, most of the fructose is taken up in the liver while the majority of the glucose passes on to the general circulation, forcing the liver to use fructose for glycolysis and lipogenesis (160, 161). In this manner, a high intake of fructose can lead to NAFLD by inducing lipid production in the liver, which can further lead to insulin resistance (161). Indirectly, a high intake of added sugars will lead to weight gain and increased fat mass, which can lead to a dysregulation of the lipid and carbohydrate metabolism and increase the risk of metabolic syndrome, insulin resistance and NAFLD (161).

There are some discrepancies in the literature regarding the diet composition of the "Western diet" and risk of developing several diseases, with the researchers using different amounts of added sugars and cholesterol, which could impact how the results are interpreted. Despite the differences in the composition of the "Western diet" in the literature, the diet has a tendency of showing similar results with weight gain, increased fat mass and hepatic lipid accumulation, which strengthens the adverse metabolic side effects from this type of diet (124, 152, 158, 159). In summary, the HFS diet induced adiposity and liver steatosis, with an increased expression of genes related to LD binding and lipogenesis, suggesting the

development of NAFLD (160, 161). Thus, the "Western diet" model of fatty liver has similarities to human NAFLD, making it a better model compared to the MCD model (124).

6.3.4 Diets with a high fat and low carbohydrate content

The two diets rich in fats and low in carbohydrates resulted in no differences in bodyweight, but caused decreased liver weight (HFK) and liver mass relative to bodyweight (HF), which is contrary to other similar studies (163, 164). The HF diet increased fat mass, especially in the inguinal area, while the HFK diet did not alter fat mass when compared to their controls. As noted earlier, a HF diet usually leads to considerable weight gain (165). The limited weight gain noted in the experiment analyzed in this master thesis seems not to be representable for the expected weight gain on such a diet (165, 166). The food being stale and a lower caloric intake may explain why the mice in these diet interventions had a lower liver weight than mice from similar studies (163, 164). To become obese and develop steatosis, the mice should be exposed to a HF and HFK diet for periods longer than 10 weeks, which could explain why the mice in the HFK group did not show any significant differences regarding bodyweight and increased liver weight, as they were only fed a ketogenic diet for 4 weeks (163, 164). Furthermore, if the fat content of the diet becomes too high and the carbohydrate content too low, some mice loose rather than gain weight, which is thought to be coupled to increased energy expenditure and could explain why the mice in the HFK group did not gain weight (167).

Neither the HF diet nor the HFK diet showed a significant increase in the amount of TAGs in liver, but both diets did show an increase in the protein expression of Plin2 and Plin3 in the western blot compared to their controls, in addition to a significant higher expression of LD binding genes, such as *Plin2, Plin5* and *Cideb* in the RT-qPCR, which is expressed on the surface of LDs and central for the amount of LDs in the liver (86, 94). These results are only preliminarily, since the data from the western blot only stems from duplicates from pooled samples. As mentioned, several pathways are included in the synthesis and oxidation of FAs, and several of these mechanisms could be responsible for the lack of hepatic lipid accumulation (142). The HFK diet consists of only 0.1% carbohydrates. Glucose is an important substrate for synthesizing TAG for storage in WAT, and the low content of carbohydrates could have an impact on the low accumulation of hepatic TAGs observed in

these mice (49). The mice had reduced expression of the transcription factors *Srebf1c* and *Chrebp*, in addition to several downstream genes related to lipid synthesis, such as *Fasn, Acc* and *Scd1*. Furthermore, mice fed the HF and the HFK diet had showed upregulated genes related to lipid oxidation, such as the transcription factor *Pparα* and downstream genes *Cpt1α* and *Acox1*. The mice fed the HFK diet had increased expression of a higher number of lipolytic genes. This indicates a higher and more effective oxidation of FAs on diets rich in fats and low in carbohydrates, in addition to a decreased production of lipids, and may result in differences in LD size. The higher expression of LD binding genes may also indicate a higher abundance of LDs (86). This is mere speculations, since no histology measurements were done.

Other studies including ketogenic diets usually have a low content of both carbohydrates and proteins, which is necessary to induce ketosis among rodents (142). The low content of protein in these diets may lead to a low natural source of methionine and choline, which have been shown to be important for liver health, as a lack of these amino acids lead to the development of NAFLD and inflammation in the liver (142). Mice fed a diet consisting of 93.3% of energy from fat and only 4.7% of energy from protein for 12 weeks actually showed a lean bodyweight and were hypoinsulinemic, but had developed NAFLD with inflammation in the liver and were glucose intolerant (142). This study is not comparable to our study, due to differences in the caloric content, as the high fat diets used in this master thesis contained 20% of calories from protein and either 60% (HF) or 80% (HFK) of calories from fat. Signs of NAFLD or inflammation was not observed among the mice included in this master thesis, which could be due to the high protein content and a sufficient content of methionine and choline in the diet (142).

7 Conclusion

Because the dietary intervention studies are not directly comparable, the results should be interpreted with caution. However, mice fed the HFS diet, which is rich in sucrose, saturated fat and cholesterol were obese and had increased accumulation of liver fat. This was not the case for the HF and the HFK diets. Mice fed a HF diet had increased fat mass in the inguinal area, but neither the HF nor the HFK diet induced liver steatosis, despite an increase in hepatic gene expression of LD binding genes. These mice had a reduced lipogenic gene expression in liver, indicating reduced lipogenesis. In addition, the mice fed the HF and HFK diets also showed an increased expression of lipolytic genes, indicating a more efficient oxidation and removal of FAs. This was not the case for the mice fed a HFS diet, possibly due to increased intake of sucrose. The combination of high content of saturated fat, cholesterol and sucrose in the HFS diet seemed to be central for accumulation of lipids in the liver.

The MCD diet resulted in a higher liver mass relative to bodyweight, in addition to accumulation of hepatic lipids and inflammation, which is indicative of NAFLD with possible fibrosis and development of NASH. In contrary to human NASH, the mice fed the MCD diet lost a substantial amount of bodyweight and fat mass, raising the question whether this is a representable model of human NAFLD where obesity is a predisposing factor. The MCD fed mice also showed a decrease in the genes related to lipid oxidation, which may indicate a decrease in the lipid oxidation despite hepatic lipid accumulation. Fasting and the MCD diet resulted in high expression of *Plin4* and *Cidec*. The biological significate of increased expression of these LD binding proteins is unknown and this novel observation warrants further investigations.

Our results suggests that the HFS diet seems to be more detrimental for liver health and unfavourable metabolism in liver, compared to fasting, the HF diet or the HFK diet. The only diet that seems worse was the MCD diet, which in addition to accumulation of lipids also showed signs of inflammation. Even though the HF diet led to an increase in fat mass, the HFS diet seemed to induce both an increased bodyweight, increases in fat mass and liver steatosis, indicating more detrimental effects on metabolic effects than a diet richer in fats and lower in carbohydrates.

7.1.1 Future directions

As mentioned, parameters such as cholesterol content in liver, glucose tolerance or insulin resistance were not measured in this master thesis. A glucose tolerance test prior to euthanasia of the animals could be used to check for insulin resistance, which further could be used to assess for metabolic syndrome among the mice, and its transferability to human metabolic syndrome. In addition, liver histology, the serum cholesterol and the glucose tolerance or insulin resistance should be measured to get a broader picture of the metabolic effects on the whole organism, liver health and liver metabolism with the different diets. In future studies, diet intervention studies should ideally be repeated and run simultaneously to reduce variability regarding different time intervals and how the diet interventions are conducted to be able to compare across all diets. The HF study used should be switched with data from a better HF diet intervention study to represent actual weight gain on such a diet.

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9 Appendix

Appendix 1. Primers used in RT-qPCR.

Appendix 2. Solutions used for Western blotting

Appendix 1. Specific primer pairs used for the RT-qPCR analyses.

Gene name	Forward primer	Reverse primer
Rplp0	TGCACTCTCGCTTTCTGGA	GCGCTTGTACCCATTGATGATG
Tbp	AGCCTTCCACCTTATGCTCAG	GCCGTAAGGCATCATTGGACT
Ppib	ACTTCATGATCCAGGGTGGAG	GCGCTCACCATAGATGCTCTT
Polr2a	CTGCGCACCATCAAGAGAGT	ACCTCCCTCCGTTGTTTCTG
Rpl32	CGCAAGTTCCTGGTCCACAA	TGTGAGCAATCTCAGCACAGT
Plin1	ACCTGGAGGAAAAGATCCCG	TTCGAAGGCGGGTAGAGATG
Plin2	GGGCTAGACAGGATGGAGGA	CACATCCTTCGCCCCAGTTA
Plin3	CGAAGCTCAAGCTGCTATGG	TCACCATCCCATACGTGGAAC
Plin4	ACCAACTCACAGATGGCAGG	AGGCATCTTCACTGCTGGTC
Plin5	GGTGAAGACACCACCCTAGC	CCACCACTCGATTCACCACA
Cidea	CCTTAAGGGACAACACGCAT	TTGCTTGCAGACTGGGACATA
Cideb	GTCTGTGATCATAAGCGGACAGT	GTGTTAGCACTCCACGTAGCA
Cidec	GCTCACAGCTTGGAGGACC	CCATCTTCCTCCAGCACCAG
Abhd5	TCCCTTTAACCCCTTGGCTG	GCCTCAAACGCTGCACTAGA
G0s2	TCTCTTCCCACTGCACCCTA	GGATCAGCTCCTGCACACTT
Lipe	TCACGCTACACAAAGGCTGC	GAGAGTCTGCAGGAACGGC
Pnpla2	CACTCACATCTACGGAGCCT	TAATGTTGGCACCTGCTTCAC
Cpt1α	CCCAGCTGTCAAAGATACCGT	GCTGTCATGCGTTGGAAGTC
Acox1	AATCTGGAGATCACGGGCACTT	GTCTTGGGGTCATATGTGGCAG
Lxrα	GACTTCAGTTACAACCGGGAAGA	ATTCATGGCTCTGGAGAACTCAAA
Lxrβ	GAAGGCGTCCACCATTGAG	AAGTCGTCCTTGCTGTAGGT
Srebf1a	GGCCGAGATGTGCGAACTG	GTTGTTGATGAGCTGGAGCATGT
Srebf1c	GGAGCCATGGATTGCACATTT	CAGCATAGGGGGCGTCAAA
Fasn	CTTCGGCTGCTGTTGGAAGTC	GTGTTCGTTCCTCGGAGTGAG
Acc	TACGCTGACCGAGAAAGCAG	GATCTACCCGACGCATGGTT

Scd1	GAGGCGAGCAACTGACTATC	GGTGGTCGTGTAAGAACTGG
PkLr	CTCTGCCTTCTGGATATCGACTC	CTCTGCCTTCTGGATATCGACTC
Chrebp	TGCAGCCCAGCCTAGATGAC	AGCTGGGGGACTCTATGTAGTT
Chrebpα	CCTCTTCGAGTGCTTGAGCC	GGATCTTGTCCCGGCATAGC
Chrebpβ	GACCCGAGGTCCCAGGAT	CACTTGGGAGAGACCAGCTT
Dgat1	CCATACCCGGGACAAAGACG	GAATCTTGCAGACGATGGCAC
Dgat2	GGCTACGTTGGCTGGTAACT	ATGGTGTCTCGGTTGACAGG
Pparα	ACTACGGAGTTCACGCATGT	GTCGTACACCAGCTTCAGCC
Pparδ	ACATGGAATGTCGGGTGTGC	CGAGCTTCATGCGGATTGTC
Pparγ	TTGCTGTGGGGATGTCTCAC	AACAGCTTCTCCTTCTCGGC
Pgc1a	AGTCCCATACACAACCGCAG	CCCTTGGGGTCATTTGGTGA
Pgc1b	ACGTGGACGAGCTTTCACTG	TTCAGAGCGTCAGAGCTTGC
Tnf	CCACCACGCTCTTCTGTCTAC	CTGATGAGAGGGAGGCCATT
Il1b	GCTGAAAGCTCTCCACCTCA	TGTCGTTGCTTGGTTCTCCT

Appendix 2. Solutions used for Western blotting

RIPA buffer

5mL 1 M Tris-HCl

3mL 5 M NaCl

4mL 25% NP-40

5mL 10% Sodium deoxycholate

0.5mL 20% SDS

4mL 0.5 M EDTE

78.5mL MilliQ water

4x Laemmli buffer

1.5 mL 1 M Tris-HCl pH 6.5

3 mL of 1 M DTT (dithiotreitol)

0.6 g of SDS (sodium deodecyl sulfate)

30 mg of Bromophenol blue

2.4 mL of Glycerol

Milli-Q water was added until the final volume reached 7.5 mL

10x Running buffer

30g 250mM Tris-base

144g 1.92 M Glycine

10g SDS (sodium deodecyl sulfate)

MilliQ water was added until the solution reached 1 L

1x Running buffer

100mL 10x running buffer

900mL dH_2O

10x TBS-T

24.2 g 200 mM Tris-base

80.15 g 1370 mM NaCl

1 M HCl

Milli-Q water was added until the solution reached 1 L

1x TBS-T

100 mL 10x TBS-T

1 mL Tween-20

Milli-Q water was added until the solution reached 1 L

Blocking buffer

5 g BSA (bovine serum albumin)

100 mL 1x TBS-T

10x Transfer buffer

30 g 250 nM Tris-base

144 g 1.92 M Glycine

Milli-Q water was added until the solution reached 1 L

1x Transfer buffer

100 mL 10x transfer buffer

800 mL Milli-Q water

100 mL Methanol