MASTERARBEIT | MASTER'S THESIS

Titel | Title

Effects of fasting and ketogenic diets on murine brain gene expression

verfasst von | submitted by

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angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien | Vienna, 2025

Studienkennzahl lt. Studienblatt | Degree programme code as it appears on the student record sheet:	UA 066 838
Studienrichtung lt. Studienblatt | Degree programme as it appears on the student record sheet:	Masterstudium Ernährungswissenschaften
Betreut von | Supervisor:	Univ.-Prof. Dr. Jürgen König

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1. Introduction

1.1. Mitochondria

Mitochondria are key organelles for cellular energy production, converting nutrients into ATP through oxidative phosphorylation. In addition to their role in energy metabolism, they contribute to biosynthetic processes, ion transport and cell motility. Mitochondria also generate reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{\bullet-}$) and hydroxyl radicals ($HO^{\bullet}$). These ROS can accumulate and lead to oxidative damage, contributing to mutations and deletions in mitochondrial DNA over time. Mitochondria possess their own DNA and can make up 20% of the cytoplasmic volume. They are also highly dynamic, moving along the cytoskeleton. They are built with an outer and inner mitochondrial membrane. In the mitochondria, electrons from NADH are transferred to oxygen through three major enzyme complexes embedded in the inner mitochondrial membrane (IMM). These enzyme complexes can also function as proton pumps to acidify the intermembrane space. The NADH dehydrogenase complex accepts electrons from NADH and passes them through cofactors to cytochrome c reductase. This second complex transfers electrons via cytochrome c to cytochrome c oxidase, which ultimately reduces molecular oxygen (O_2) to water (H_2O). This electron transfer not only drives the reduction of O_2 to H_2O, but also establishes a proton gradient essential for ATP production (Alberts et al., 2022).

1.1.1. Mitochondria and oxidative stress

Oxidative stress refers to an imbalance between the production of ROS and their elimination by cellular defense systems, including enzymatic antioxidants such as superoxide dismutases (SOD) and glutathione peroxidase (GPX) (Milani et al., 2013). Oxidative stress is primarily caused by ROS from the mitochondrial respiratory chain, especially at Complexes I and III. Cellular oxidative Stress is linked to the development of many diseases and accelerates the aging process (Lenaz et al., 2002). Mitochondria are both major sources and primary targets of ROS, which can damage mitochondrial DNA and impair energy production over time, as proposed by the mitochondrial theory of aging (Harman, 1956). To control ROS in the cells, there are different pathways. One of them is the enzymatic antioxidant pathway. There $O_2^{\bullet-}$ from oxidative metabolism are neutralized in two steps. First, SOD converts the radicals, then GPX and/or catalase (CAT) break down H_2O_2 into H_2O. The antioxidants of both steps need to be in a balance, to ensure cellular homeostasis (De Haan et al., 2003; Zhao, Wang, et al., 2022).

1.2. Ketogenic diet

Ketogenic diets (KDs) have gained attention for their potential health benefits like weight loss (Bueno et al., 2013; Patikorn et al., 2023) and improved seizure control in patients with epilepsy (Lefevre & Aronson, 2000). By shifting the body's energy production toward ketogenesis and fatty acid oxidation, they may have significantly improvements in glycemic and lipid profiles. KDs are characterized by very low carbohydrate intake, not more than 50 g/day, and increased fat and protein consumption, which promote the production of ketone bodies as an alternative energy source (V. J. Miller et al., 2018; Patikorn et al., 2023). KDs typically raises blood levels of ketone bodies (KB): β-hydroxybutyrate, acetoacetate and acetone (V. J. Miller et al., 2018). KBs are constantly produced in the liver, with production increasing when cells rely primarily on fat due to limited glucose availability. Once formed, KBs circulate in the blood, serving as alternative fuel for tissues like the heart, muscles and brain, or are excreted via urine and breath (Masi et al., 2022; Newman & Verdin, 2014). The main KBs, acetoacetate and β-hydroxybutyrate, serve as alternative energy sources for the brain, independently of glucose, whereas medium-chain fatty acids (MCFA) can be utilized by the brain only to a limited extent. (Han et al., 2021). The status of ketosis can be induced under non-fasting conditions either by KDs or supplementation with MCFA and exogenous ketone esters or salts (Jensen et al., 2020). KDs may include both long-chain triglycerides (LCT), which contain fatty acids > 12 carbon atoms, and medium-chain triglycerides (MCT) consisting of fatty acids of 6-12 carbon atoms (Augustin et al., 2018; Sternberg et al., 2023). MCTs are hydrolyzed into MCFAs, whereas LCTs yield long-chain fatty acids (LCFAs). MCFAs are directly transported to the liver and can enter mitochondria without the carnitine shuttle due to their smaller size. They are rapidly oxidized through ß-oxidation and not stored as fat. However, they lack essential fatty acids such as omega-3 and omega-6. In contrast, LCFAs must be packaged into micelles and transported via the lymphatic system, resulting in slower metabolism. Due to these properties, MCTs are considered more ketogenic (Augustin et al., 2018; Dunn et al., 2023; Nimbkar et al., 2022). MCTs demonstrate a wide array of positive effects, including support for weight reduction (Mumme & Stonehouse, 2015) and enhancement of mitochondrial and antioxidant function (Han et al., 2021).

1.2.1. MCTs in the brain

As previously described MCTs are quickly broken down into MCFAs during digestion, transported to the liver and rapidly metabolized via β-oxidation producing ketones as energy source. While the brain is primarily fueled by glucose, and to a lesser extent by KBs, MCFAs can cross the blood-brain barrier and reach significant levels in the brain offering an additional energy source for brain cells. There is evidence that MCFAs can directly influence brain cell energy metabolism in distinct ways. KDs with MCT supplementation have been shown to improve seizure control in children and adolescents with epilepsy and may benefit brain energy metabolism, making them a potential therapy for cancer, Alzheimer's disease or diabetes (Augustin et al., 2018). Moreover, MCTs show improvements regarding cognitive functions and neuroprotection. In a recent study by Sun et al. (2024) MCTs, alone and in combination with docosahexaenoic acid (DHA), were investigated for their effects on cognitive decline in mice. The results demonstrated that MCT supplementation, either alone or in combination with DHA significantly delayed age-related cognitive decline compared to the control group. These findings underline the neuroprotective and cognition-supporting properties of MCTs, particularly in the context of aging (Sun et al., 2024).

1.3. Intermittent fasting and caloric restriction

Intermittent fasting (IF) and caloric restriction (CR) continue to be extensively studied for their potential metabolic benefits. Growing evidence indicates that IF or CR triggers adaptive autophagy, which may contribute to enhanced longevity (Shabkhizan et al., 2023). IF is characterized by alternating periods of fasting and consumption. It has been shown that IF can improve lipid profiles, contribute to the reduction of overweight and obesity, protect against inflammation and exert beneficial effects on mitochondrial function and oxidative balance. IF and CR show strong potential in reducing oxidative stress and promoting longevity by enhancing antioxidant defenses and upregulating protective enzymes such as Nuclear factor erythroid-derived 2-like 2 (NRF2). These effects contribute to reduced obesity and dyslipidemia, as well as enhanced cardiovascular protection and potentially increased longevity (Diab et al., 2024; Varady et al., 2022). Energy restriction paradigms have been shown to reduce oxidative stress in the brain, which may contribute to improved cognitive function and structural brain integrity. It promotes a metabolic shift from glucose to lipid utilization, thereby enhancing mitochondrial function. These effects are supported by improved mitochondrial metabolism,

increased biogenesis, and stabilized mitochondrial dynamics, involving key regulatory pathways such as NRF2 (L. Li et al., 2013; Zhao, Jia, et al., 2022).

1.4. Nuclear factor erythroid-derived 2-like 2

NRF2 is a key nuclear factor that regulates the expression of various genes involved in detoxification, drug transport, apoptosis prevention and protein degradation. Its activation and suppression play an important role in protecting cells from oxidative stress and diseases such as cancer (Niture et al., 2014). Under normal conditions, NRF2 is constantly marked for degradation by Kelch-like ECH-associated protein 1 (KEAP1). KEAP1 forms a complex with the Cullin3 E3 ubiquitin ligase, which tags NRF2 with ubiquitin molecules. This signals NRF2 for destruction by the proteasome. As a result, NRF2 activity remains low when the cell is not under stress. However, during oxidative stress, this interaction between KEAP1 and NRF2 is disrupted. KEAP1 can no longer direct NRF2 to degradation, allowing NRF2 to accumulate and move into the nucleus. Inside the nucleus, NRF2 forms a complex with small musculoaponeurotic fibrosarcoma (Maf) proteins, which is necessary to activate specific genes. These genes contain a sequence called the antioxidant response element (ARE). By binding to AREs, the NRF2-Maf complex triggers the expression of protective genes, many of which are involved in antioxidant defense, detoxification and maintaining cellular homeostasis (Niture et al., 2014; Pouremamali et al., 2022). NRF2 plays a complex role in various diseases, with both protective and potentially detrimental effects. Impaired NRF2 function has been linked to depression. The modulation of NRF2 may offer therapeutic benefits (Sani et al., 2023). Additionally, NRF2 activators show promise in alleviating autism-like behaviors by counteracting oxidative stress, inflammation, and mitochondrial dysfunction (J. Yang et al., 2020). However, in pancreatic cancer, mutations or deletions of KEAP1 lead to persistent NRF2 activation, shifting its role from tumor suppression to tumor promotion by enhancing tumor growth, metastasis, and chemoresistance. While NRF2 activators may help prevent tumorigenesis, they can also contribute to chemoresistance, whereas NRF2 inhibitors have been shown to suppress tumor progression and improve chemotherapy sensitivity (Qin et al., 2019).

1.5. Targets of MCFA metabolism

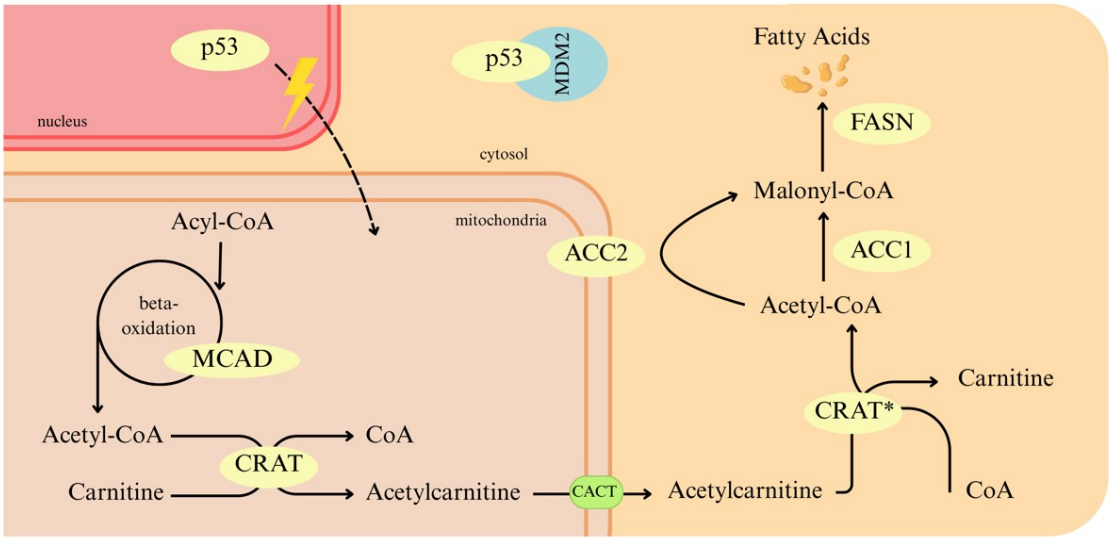

Figure 1.1 **Schematic representation of lipid metabolism pathways and p53 within the mouse cell.** The illustration highlights the roles of ACC1 and ACC2, FASN, CRAT and MCAD, emphasizing their cellular localization and functional interplay in lipid homeostasis. Under normal conditions, p53 is retained in the cytosol through interaction with MDM2. In response to oxidative stress, p53 translocates to the mitochondria. ACC1/2 – Acetyl-CoA carboxylase 1/2, FASN – Fatty acid synthase, CRAT – Carnitine O-acetyltransferase, MCAD – Middle-chain acetyl-CoA dehydrogenase, CACT - carnitine/acetylcarnitine translocase, CRAT* - cytosolic Crat, p53 – tumor suppressor protein p53.

1.5.1. Acetyl-CoA carboxylase

Acetyl-CoA carboxylases (ACCs) are important factors in the energy metabolism. ACCs are enzymes that function as catalysts for the carboxylation of acetyl-CoA, leading to the formation of malonyl-CoA, an essential intermediate in the regulation of fatty acid metabolism. Malonyl-CoA acts as C2 donor for the de novo synthesis of long-chain fatty acids to form very long-chain fatty acids in adipose tissues. This reaction is catalyzed by the fatty acid synthase (FASN) (Abu-Elheiga et al., 2005). ACCs can occur in two isoforms: ACC1 and ACC2. Whereas the alpha enzyme ACC1 is encoded by the *Acaca* gene, the beta isoform ACC2 is encoded by *Acacb* (Abu-Elheiga et al., 2005; Y. Wang et al., 2022). ACC1 is mainly found in the cytosol of lipogenic tissues like liver and adipose (Munday, 2002). It regulates the production of medium- and long-chain fatty acids. While ACC1 converts acetyl-CoA to malonyl-CoA and consequently into fatty acids with FASN as catalyzer (Y. Wang et al., 2022), the pathway of ACC2 is slightly different. ACC2 is localized in the outer mitochondrial membrane (OMM) in oxidative tissues such as heart and skeletal muscle (Munday, 2002). It also converts acetyl-CoA into malonyl-CoA, which does not enter de novo fatty acid synthesis. Instead ACC2 regulates fatty acid β-oxidation

by inhibiting carnitine palmitoyltransferase 1 (CPT1), which also is localized in the OMM. CPT1 is the key enzyme for mitochondrial LCFA transport. Malonyl-CoA binds allosterically to CPT1, reducing its activity and thereby regulating mitochondrial fatty acid β-oxidation (Y. Wang et al., 2022).

1.5.2. Middle-chain acetyl-CoA dehydrogenase

Acetyl-CoA Dehydrogenases (ACAD) serve an important function in the degradation of fat and protein. The *Acadm* gene encodes the enzyme medium-chain acyl-CoA dehydrogenase (MCAD). MCAD catalyzes the first step of the mitochondrial β-oxidation (Koster et al., 2014; Shen et al., 2009). Five ACAD subfamilies are involved in the β-oxidation of fatty acids and each show preferential activity for acyl-CoA substrates of certain chain lengths. While *Acadm* encodes medium-chain acyl-CoA, other enzymes of the subfamily encode small-chain- (ACADS), long-chain- (ACADL) or very long-chain- (ACADV/ACADV2) acyl-CoA dehydrogenases. In eukaryotes, these mitochondrial proteins perform comparable biochemical functions by oxidizing various acyl-CoA molecules, generated from fat and protein breakdown, into enoyl-CoA. MCAD metabolizes octanoyl-CoA (C8) into enoyl-CoA (Shen et al., 2009). *Acadm* gene mutations lead to medium-chain acyl-CoA dehydrogenase deficiency. Individuals with this metabolic disorder struggle to break down fatty acids efficiently, leading to energy deficits, especially during fasting. This impaired metabolism increases their vulnerability to catabolic stress, increasing the risk of hypoketotic hypoglycemia, which can result in coma or even death during periods of illness (Madeira et al., 2023; Wilcken et al., 2007).

1.5.3. Carnitine O-acetyltransferase

Acylcarnitines, formed by the conjugation of fatty acids with L-carnitine, are important players in cellular energy metabolism and are widely utilized in these processes (Dambrova et al., 2022). Carnitine acyltransferases including carnitine O-acetyltransferase (CRAT), CPT1 and CPT2 as well as carnitine octanoyltransferase (CROT) are essential for CoA homeostasis by converting acyl-CoA esters into acyl-carnitine derivatives that can cross cellular membranes (Westin et al., 2008). This process prevents excess acetyl-CoA accumulation, supports metabolic flexibility, and maintains the equilibrium between fatty acid- and glucose oxidation. CRAT is localized in the mitochondria, as well the peroxisome, the endoplasmatic reticulum (ER) and the nucleus (Dambrova et al., 2022; Laera et al., 2020). It interacts with the mitochondrial carnitine shuttle, including CPT1 and CPT2 and the carnitine/acetylcarnitine

translocase (CACT) but also with enzymes of the β-oxidation to coordinate transport of acyl groups between acetyl-CoA and carnitine. CACT, encoded by *Slc25a20*, is located on the IMM and enables the exchange of acetylcarnitine and free carnitine (Laera et al., 2020; Volpicella et al., 2025). Acylcarnitines are classified into four categories based on their chain length: short-chain (C2–C5), medium-chain (C6–C12), long-chain (C13–C20), and very long-chain acylcarnitines (>C21) (Dambrova et al., 2022). CRAT primarily mediates the transfer of short-chain acyl groups, like acetyl-L-carnitine, but is also capable of efficiently synthesizing acylcarnitines with carbon chain lengths of up to C8 and C10 (Dambrova et al., 2022; Jones et al., 2010). Whereas CROT, which are localized in the peroxisome (Dambrova et al., 2022), governs the fatty acid oxidation of very long-chain acylcarnitines (Volpicella et al., 2025). CRAT plays a role beyond lipid metabolism by also regulating amino acid catabolism. It helps process acetyl-CoA from amino acid degradation, preventing mitochondrial dysfunction. Its dysregulation is linked to metabolic disorders like obesity, insulin resistance (Volpicella et al., 2025), and mitochondrial diseases such as carnitine acetyltransferase deficiency (Laera et al., 2020). Chronic overnutrition impairs CRAT function by inhibiting its activity through lipid accumulation. Increased fatty acid uptake into mitochondria reduces CRAT-derived acylcarnitine production and export, disrupting metabolic flexibility in skeletal muscle, liver, heart and pancreas. Moreover, obesity and diabetes decrease CRAT specific activity at a posttranslational level (Seiler et al., 2014).

1.5.4. Fatty acid synthase

FASN serves an essential function in the production of fatty acids inside the cell through de novo fatty acid synthesis. FASN uses NADPH as a reducing cofactor to convert acetyl-CoA and malonyl-CoA into palmitate (C16), which then serves as a precursor for the synthesis of more complex fatty acid metabolites (Fafián-Labora et al., 2019; Xiao et al., 2024). In healthy human tissues, this process is usually suppressed because cells prefer to use external lipids for building membranes, keeping FASN levels low. However, during senescence, FASN levels increase. Blocking FASN prevents this aging process by reducing the energy supply from mitochondria (Fafián-Labora et al., 2019). When there is an excess of glucose, FASN helps convert glucose into fatty acids. These fatty acids are then either stored as triglycerides within lipid droplets or released into the bloodstream as very low-density lipoproteins (VLDL) (Fhu & Ali, 2020). FASN is essential for immune cell function. As a result, it influences the development and progression of various diseases, such as infections, cardiovascular disorders, inflammatory and autoimmune conditions, as well as cancer (Xiao et al., 2024). FASN is a key enzyme in lipid

metabolism that enables tumor cells to adapt their energy supply to meet high demands. Additionally, FASN-positive tumor signatures may serve as predictive biomarkers for identifying patients who could benefit from FASN-targeted therapie. FASN is crucial for brain development and lipid metabolism in neural stem cells, which play an important role in both early brain formation and lifelong brain function. These cells continuously produce new neurons, helping the brain adapt and remain functional (Fhu & Ali, 2020).

1.5.5. Tumor suppressor protein p53

p53 is a key tumor suppressor protein that regulates processes like cell cycle, DNA repair, and apoptosis. It responds to stress signals by altering expression of various genes. p53 is mutated or inactive in many cancers, which results in the loss of its protective role (Temaj et al., 2024). Therefore mutations in *Trp53*, the gene which encodes p53 in mice, or its regulatory pathways can disrupt p53 function and contribute to cancer development (Lahalle et al., 2021). p53 responses to nucleolar stress caused by impaired ribosome biogenesis. Under normal conditions, murine double minute 2 protein (MDM2), a key negative regulator of p53, continuously promotes its degradation to keep p53 levels low (Nicolas et al., 2016; Hároníková & Vojtěšek, 2018). When ribosome production is disrupted, unassembled ribosomal components, such as the 5S ribonucleoprotein, accumulate and bind to MDM2. This prevents MDM2 from degrading p53, leading to its stabilization. Activated p53 then initiates cell cycle arrest or apoptosis to maintain cellular integrity (Nicolas et al., 2016; Temaj et al., 2024). Additionally, MDM2 regulates the electron transport chain in the mitochondria and helps with mitochondrial mitophagy. The p53 pathway influences among others the glucose- and pyruvate-metabolism in the cell (Lahalle et al., 2021) As previously shown p53 activity induces extensive tissue remodeling in adipose tissue during IF. Loss of p53 in adipocytes boosts the metabolic benefits of IF, including greater weight loss, improved insulin sensitivity, and reduced inflammation, indicating potential therapeutic value (Reinisch et al., 2024).

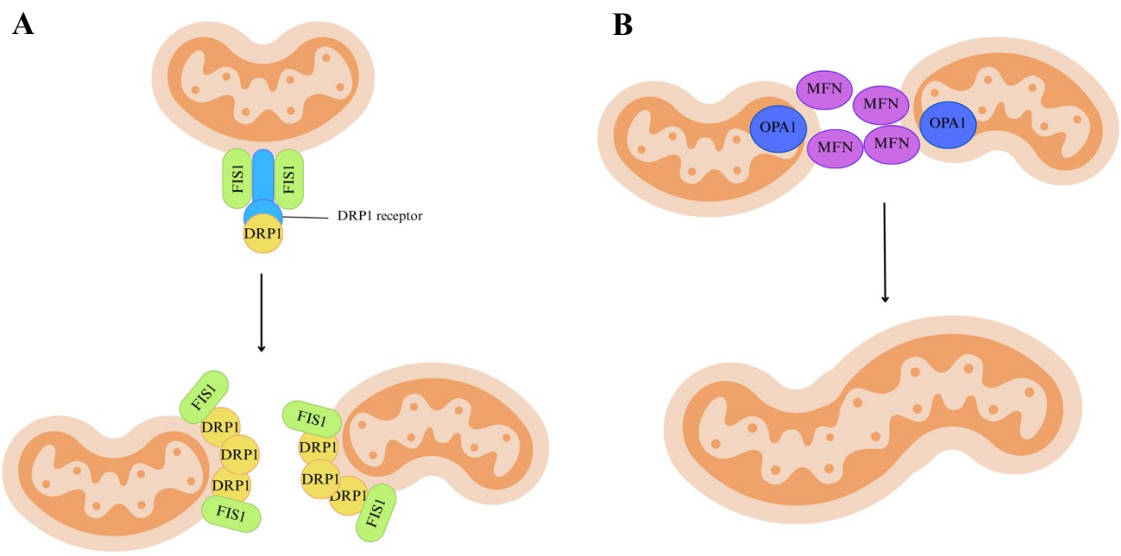

Figure 1.2 **Mitochondrial fission and fusion dynamics. (A)** During Fission, FIS1 acts as an adaptor protein that recruits DRP1. **(B)** MFN1 and MFN2 mediate the fusion of the OMM, while OPA1 regulates IMM fusion. Drp1 – dynamin-related protein 1, Fis1 – fission 1 protein, Mfn1/2 – Mitofusin 1/2, Opa1 – optic atrophy 1.

1.5.6. Mitochondrial fission 1 protein

The mitochondrial fission 1 protein (FIS1) is a regulator of mitochondrial dynamics. It is located on the OMM. FIS1 is essential for mitochondrial fission. It recruits important proteins, including the cytosolic dynamin-related protein 1 (DRP1), which is necessary for this process. For this purpose, DRP1 accumulates at the division site in such a way that a ring-like structure forms around the organelle and separates it (Grel et al., 2023; Serasinghe & Chipuk, 2016). Another protein that can regulate DRP1 is the mitochondrial fission factor (MFF). In absence of FIS1 and MFF also the dynamic proteins of 49 (MiD49) and 51 kDa (MiD51) can recruit DRP1. (Rovira-Llopis et al., 2017). Additionally, FIS1 interacts with other proteins involved in mitochondrial dynamics, such as mitofusin-1 (MFN1), mitofusin-2 (MFN2) and optic atrophy 1 (OPA1), to maintain a balance between fission and fusion. Maintaining a balance is crucial for cellular homeostasis and neuronal function. Disruptions in key proteins like DRP1, FIS1 are linked to neurodegenerative diseases (NDs) (Grel et al., 2023), as well as the development of insulin resistance and type 2 diabetes (Rovira-Llopis et al., 2017) and sarcopenia (Marzetti et al., 2013). Li et al. recently showed that IF diet significantly downregulates the expression of murine Fis1 and Mff (Y. Li et al., 2023).

1.5.7. Mitofusin-1 and Mitofusin-2

Mitochondrial fusion is regulated by various proteins, such as MFN1, MFN2 and OPA1. Mitofusin proteins (MFNs) reside in the OMM. MFN1 proteins can connect mitochondria by interacting across their membranes, bringing two opposing mitochondria together. MFN2 is responsible for linking the ER to mitochondria, a connection for proper calcium (Ca^{2+}) signaling (Rovira-Llopis et al., 2017). MFN1 and MFN2 work together to facilitate mitochondrial fusion by connecting the OMM and supporting IMM fusion. Their ability to form homo- and hetero-oligomers ensures proper mitochondrial communication, integrity and network stability (Grel et al., 2023; Hoppins et al., 2011). During mitophagy, a process that removes damaged mitochondria, MFN1 and MFN2 must be degraded to prevent fusion with healthy mitochondria. This allows damaged mitochondria to be divided into smaller fragments, which can then be efficiently enclosed by autophagosomes and recycled (Rovira-Llopis et al., 2017). To maintain overall balance of MFNs, the loss of Mfn1 leads to an increase in Mfn2 expression, while the absence of Mfn2 results in higher Mfn1 levels (Saita et al., 2016). Dysregulations in MFN1 and MFN2 are connected to NDs like Alzheimer's disease (Grel et al., 2023; Pahlavani, 2023; Saita et al., 2016). There is evidence that type 2 diabetes can lead to decreased expression of MFN2 (Rovira-Llopis et al., 2017).

1.5.8. Optic atrophy 1

In addition to MFN, OPA1 is important in regulating mitochondrial dynamics. OPA1, mainly situated in the IMM, plays a vital role in maintaining mitochondrial structure, function, and cellular balance by regulating IMM fusion. It supports the mitochondrial network by coordinating cristae morphology and facilitating the exchange of components, which helps preserve mitochondrial integrity and overall efficiency of the Mitochondria (Grel et al., 2023; R. Wang et al., 2021). OPA1 exists in long (l-OPA1) and short (s-OPA1) forms. It is transported into mitochondria using a mitochondrial targeting sequence (MTS). Once inside the mitochondrial matrix, this sequence is removed by the mitochondrial processing peptidase (MPP). The protein then exits through the translocase of the inner membrane (TIM), which is as transport channel, laterally and is incorporated into the IMM as the l-OPA1. To provide proper regulation of IMM fusion, short and long OPA1 must cooperate (Saita et al., 2016; R. Wang et al., 2021). Dysfunctions of OPA1 can impair mitochondrial fusion by affecting its interaction with MFN, leading to cristae alterations and mitochondrial defects linked to NDs (Grel et al.,

2023; Hoppins et al., 2011). IF diet was shown to upregulate OPA1 expression in murine tissues (Y. Li et al., 2023)

1.6. Targets of antioxidative response

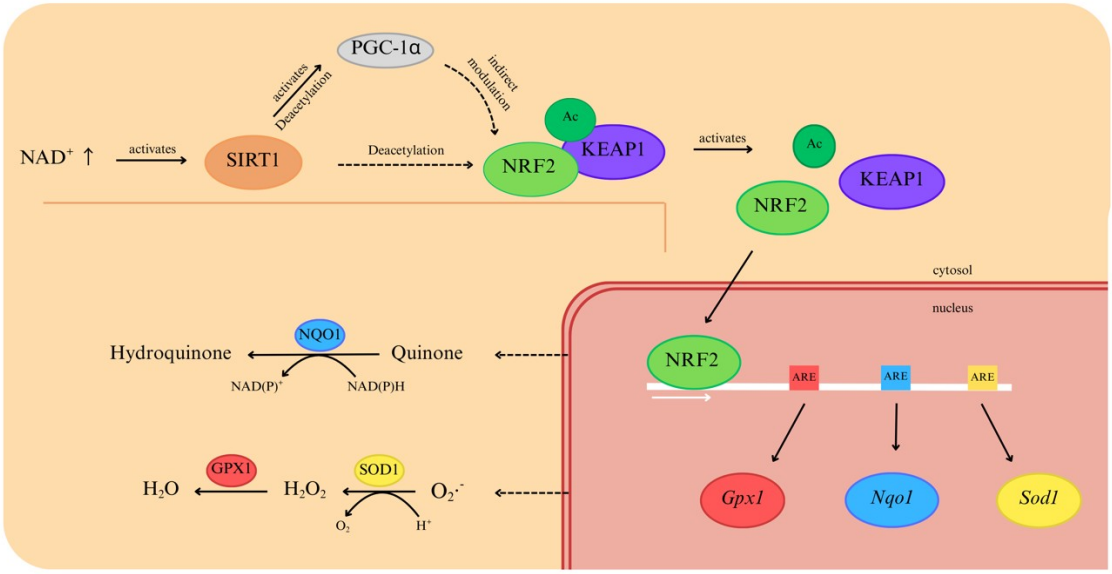

Figure 1.3 **NRF2-mediated transcriptional activation of antioxidant genes.** Upon activation, SIRT1 promotes NRF2 nuclear translocation. In the nucleus, NRF2 binds to AREs, inducing transcription of *Nqo1*, *Ho1*, *Sod1* and *Gpx1*. The corresponding proteins act in the cytoplasm to reduce oxidative stress. Ac – Acetylation, ARE – antioxidant response element, GPX1 – glutathione peroxidase 1, KEAP1 – Kelch-like ECH-associated protein 1, NQO1 - NAD(P)H:quinone acceptor oxidoreductase 1, NRF2 – Nuclear factor erythroid-derived 2-like 2, SIRT1 – Sirtuin 1, SOD1 – superoxide dismutase.

1.6.1. Glutathione peroxidase 1

GPX1 is ubiquitously expressed and localized in the mitochondria and cytosol (De Haan et al., 2003; Handy & Loscalzo, 2022). It is a selenoenzyme that utilizes glutathione as a reducing equivalent to detoxify hydrogen peroxide and organic peroxides, thereby protecting cells from oxidative stress and exerting antioxidative effects. During each reaction, GPX1 uses two molecules of reduced glutathione (GSH) to produce one molecule of oxidized glutathione (GSSG) and H_2O (Handy & Loscalzo, 2022; Zhao, Wang, et al., 2022). The ratio of GSH to GSSG serves as a reliable indicator of oxidative stress. The ratio in resting cells is 100/1. In cells exposed to oxidative stress, this ratio can increase and reaching values as high as 1/1 (Chai et al., 1994; Zitka et al., 2012). A prolonged upregulation of GPX1 can disrupt this redox balance, leading to reductive stress and potentially enabling tumor cell survival by lowering ROS levels.

Elevated *GPX1* expression in humans has been proposed to exert antiapoptotic effects in certain cancer types by modulating redox-sensitive signaling pathways. The proper balance of GPX1 activity is essential for supporting physiological function and preventing disease progression (Handy & Loscalzo, 2022). Studies using murine transgenic and knockout models show that an imbalance between superoxide dismutases and peroxidases often leads to elevated H_2O_2 or lipid hydroperoxide levels, contributing to pathophysiological effects, particularly in tissues with low levels of second-step antioxidants (De Haan et al., 2003). GPX1 is associated with the development and progression of various cancer types like bladder, breast or lung cancer, therefore it is a promising prognostic biomarker (Zhao, Wang, et al., 2022).

1.6.2. Sirtuin 1

Silencing information regulator 2 related enzyme 1 (Sirtuin 1/SIRT1) is a deacetylase that uses NAD^+ as a cofactor to deacetylate target proteins such as Nuclear Factor kappa-light-chain-enhancer of activated B cells (NFκB), peroxisome proliferator-activated receptor gamma-coactivator-1 α (PGC-1α) and p53 (Finkel et al., 2009; Gillum et al., 2011). Its expression and enzymatic activity are enhanced under conditions of caloric restriction, a physiological state associated with improved insulin sensitivity, increased adiponectin levels and reduced inflammation, making SIRT1 a potential target for type 2 diabetes therapy (Gillum et al., 2011; Kitada & Koya, 2013). Beyond its role in glucose metabolism and pancreatic insulin secretion, SIRT1 also regulates hepatic lipid homeostasis by activating AMPK and PGC-1α, two key regulators of energy balance particularly relevant under conditions of caloric restriction (Finkel et al., 2009; Gillum et al., 2011). The upregulation of SIRT1 appears to have beneficial effects in reducing NDs like Alzheimer's disease, Parkinson's disease and Huntington's disease. Reduced *SIRT1* expression in humans is commonly connected to oxidative stress, mitochondrial dysfunction, and age-associated diseases (Manjula et al., 2021). Animal research points to the potential of sirtuin pathway modulation in supporting cognitive health and possibly preventing the onset of dementia (Matysek et al., 2023). Multiple cellular pathways interact with SIRT1, reflecting its central role in coordinating diverse physiological processes. Among these, the NFκB signaling pathway is of relevance, as its persistent activation is associated with inflammation and accelerated aging. SIRT1 contributes to longevity by negatively regulating NF-κB activity (Chen et al., 2020; Yeung et al., 2004). Meta-analyses claim that there is a correlation between higher SIRT1 levels and the occurrence of severe inflammation (Sun et al., 2024). The serine/threonine kinase AMPK represents another crucial signaling pathway involved in the regulation of energy metabolism. AMPK activates SIRT1 by increasing cellular

NAD^+ levels, which promotes the deacetylation of PGC-1α. This activation also delays cellular aging by stimulating autophagy. Thus, targeting the AMPK-SIRT1 axis may offer a promising strategy to combat age-related diseases and promote longevity (Chen et al., 2020; Zhang et al., 2014). One of the main regulators of mitochondrial health is the transcriptional coactivator PGC-1α, which supports mitochondrial biogenesis and function. SIRT1 influences these processes by deacetylating PGC-1α, thereby enhancing its activity (Chen et al., 2020; Kitada et al., 2019).

1.6.3. NAD(P)H:quinone acceptor oxidoreductase 1

NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1) is a ubiquitously expressed FAD-dependent enzyme that catalyzes two-electron reductions of quinones and related compounds using NADH or NADPH and therefore exerts major antioxidant effects. Under caloric restriction NQO1 expression is enhanced through activation of the KEAP1/NRF2/ARE signaling pathway. NRF2 binds to AREs in the nucleus, inducing the transcription of NQO1 and other cytoprotective genes (Dinkova-Kostova & Talalay, 2010; Pearson et al., 2008; Sani et al., 2023). In cancer cells Keap1 is deleted or minimally expressed, leading to persistent NRF2 activation. This drives tumor growth and metastasis (Qin et al., 2019). Meta-analyses show that different polymorphisms of NQO1 can have a considerable impact on the risk of esophageal cancer (Yanling et al., 2013) and breast cancer (Peng et al., 2014).

1.6.4. Superoxide dismutase 1

SOD1 is an antioxidant enzyme that catalyzes the dismutation of $O_2^{\bullet-}$ into molecular oxygen (O_2) and H_2O_2 via redox reactions (Eleutherio et al., 2021). SODs comprise three distinct families of enzymes, characterized by their metal cofactors: Cu/Zn-SODs, Mn/Fe-SODs, and Ni-SODs, which differ both in catalytic metal usage and in protein structure, yet all share the same function in cellular antioxidant defense. SOD1 belongs to the Cu/Zn-SOD family and is primarily localized in the cytoplasm, but has also been detected in the nucleus, lysosomes, peroxisomes and the mitochondrial intermembrane space (A.-F. Miller, 2012; Y. Wang et al., 2018). SOD1 is a downstream target of NRF2. During the early stages of pancreatic tumorigenesis, NRF2 activation induces the expression of detoxifying and antioxidant genes, including *SOD1* (Nguyen et al., 2003; Qin et al., 2019). Mutations in the *SOD1* gene are associated with inherited forms of amyotrophic lateral sclerosis (ALS) (Fukai & Ushio-Fukai, 2011). In a randomized controlled trial, Merra et al. reported a significant downregulation of

SOD1 gene expression after a very low-calorie ketogenic diet. The authors hypothesized that reduced inflammation and improved glycemic control during weight loss may be linked to a more efficient oxidative stress response (Merra et al., 2017).

1.6.5. Peroxisome proliferator-activated receptor gamma coactivator 1-*α*

PGC-1α, encoded by the *Ppargc1a* gene in mice, plays a significant role in regulating mitochondrial transcription. It regulates mitochondrial biogenesis by activating NRF2 and therefore provides a vital part in antioxidant defense. Additionally, PGC-1α participates in network dynamics and supports the autophagic clearance of damaged mitochondria (Salazar et al., 2020; Scarpulla, 2011) PGC-1α deficiency is linked to increased oxidative stress and cellular senescence, partly due to impaired autophagy and lysosomal dysfunction. Overexpression of PGC-1α can partially reverse these effects, potentially through upregulation of antioxidant genes (Salazar et al., 2020). KBs derived from KDs support mitochondrial biogenesis, dynamics, and function in neurons and fibroblasts exposed to oxidative stress, partly by upregulating *Ppargc1a*. Hasan-Olive et al. (2019) demonstrated that *Ppargc1a* expression is increased in mice fed a KD (Hasan-Olive et al., 2019).

1.7. Hypothesis

In the present study, we hypothesize that the addition of MCTs to a LCT-based ketogenic diet is associated with changes in the gene expression involved in MCFA metabolism and mitochondrial dynamics in the hypothalamus. Furthermore, we investigate whether these gene expression changes are modulated by fasting-related dietary conditions, specifically IF and CR. Additionally, the NRF2 signaling pathway has attracted growing scientific attention due to its essential role in protecting cells from oxidative stress. We hypothesize that cortical expression patterns of NRF2 downstream targets and related regulatory genes reflect potential changes in antioxidant defense and redox homeostasis in response to these dietary interventions.

1.8. Aim of the study

This master thesis aimed to explore whether the supplementation of a LCT-based ketogenic diet with MCTs is associated with

- potential changes in the gene expression involved in MCFA metabolism in the hypothalamus, and
- indications of modulation in antioxidant defense mechanisms, particularly the expression of NRF2 downstream targets, in the cortex.

In addition, the study aimed to examine whether similar effects on gene expression occur under fasting-related dietary conditions (IF, CR). Gene expression was measured in hypothalamus and cortex. We analyzed gene expression in the hypothalamus for key enzymes involved in lipid metabolism (*Acaca, Acacb, Acadm, Crat, Fasn*) and mitochondrial dynamics (*Fis1, Mfn1, Mfn2, Opa1*), as well as for the tumor suppressor gene *Trp53*, including two isoforms and *Ppargc1a*, which governs mitochondrial biogenesis. In the cortex, we further analyzed the expression of antioxidant defense-related genes, focusing on direct downstream targets of the NRF2 pathway (*Gpx1, Nqo1, Sod1*), to assess potential changes of the diets on oxidative stress regulation. Additionally, we looked at indirect regulators of the NRF2 pathway (*Ppargc1a*, *Sirt1*) to gain further insight into the modulation of cellular redox homeostasis. Gene expression data were normalized using *Rpl4* and *Hprt* as housekeeping genes, ensuring accurate comparison across samples. The observed patterns may suggest diet-related effects. However, further investigations would be required to confirm these changes and establish functional relevance.

2. Material and Methods

2.1. Materials

2.1.1. Mice diets

The product number of the mice diets are indicated in the table below.

Table **1**. Mice diets

Product number	**Diet**	**Company**
S9139-E028	SD	ssniff
S9139-E025	LCT	ssniff
S9139-E032	LCT/MCT	ssniff
V53x R/M-H auto	CR and IF	ssniff

2.1.2. Kits and equipment

Standard laboratory equipment such as plates, tubes, and pipettes were supplied from Eppendorf, VWR, Liebisch and Thermo Fisher Scientific.

Table 2. Assay kits and solvents

Kit	**Company**
HIScript III RT SuperMix for qPCR (+gDNA wiper)	Vazyme
ChamQ Universal SYBR qPCR Master Mix	Vazyme
RNase AWAY	Thermo Fisher Scientific
RNase-free ddH_2O	Vazyme
Chloroform	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Glycogen	Thermo Fisher Scientific
Ethanol 70%	Sigma-Aldrich

2.1.3. Primer

All primer pairs were ordered from Microsynth and prepared according to the manufacturer's protocol.

Table 3.1. Primer pairs

Primer	Direction	Sequence 5' to 3'	Tm [°C]	GC [%]	Primer Length [bp]	Amplicon Length [bp]
mAcaca (2)	forward	GCCGCCAGCCTGAGTTCTT	62,9	63,2	19	112
	reverse	TGGCCAACGGAGATGGTTCA	61,8	55,0	20	
mAcacb	forward	CTACAAGACGGCGCAGGTCA	62,5	60,0	20	128
	reverse	AGGCGCCAAACTTCAGCATC	61,6	55,0	20	
mAcadm (2)	forward	GGGGGAAAGGCCAACTGGTAT	62,1	57,1	21	151
	reverse	AGCATCGCTGGCCCATGTTT	63,1	55	20	
mCrat	forward	TGACTCGGCGACCTTGAACCTA	63,4	54,6	22	168
	reverse	CCCAGGGGTTTTACCACGGTTC	63,4	59,0	22	
mFasn (1)	forward	AACACGATCTGGTGCTGCCC	63,1	60,0	20	105
	reverse	TGGGGGCTGTCGTGTCAGTA	63,0	60,0	20	
mFis1	forward	GCTGGTTCTGTGTCCAAGAGCA	63,0	54,6	22	144
	reverse	GACATAGTCCCGCTGTTCCTCT	61,5	54,6	22	
mGpx1	forward	CCTGACATAGAAACCCTGCTGT	60,0	50,0	22	210
	reverse	TTCATTAGGTGGAAAGGCATC	55,9	42,9	21	
mHprt (1)	forward	CAAACTTTGCTTTCCCTGGT	56,7	45,0	20	101
	reverse	TCTGGCCTGTATCCAACACTTC	60,0	50,0	22	
mMfn1	forward	CCAGGTACAGATGTCACCACAG	60,4	54,6	22	149
	reverse	TTGGAGAGCCGCTCATTCACCT	64,0	54,6	22	
mMfn2	forward	TCCCTCTGACACCTGCCAAC	61,8	60,0	20	155
	reverse	TGCCTTCCACACCACTCCTC	61,8	60,0	20	

Table 3.2. Primer pairs

Primer	Direction	Sequence 5' to 3'	Tm [°C]	GC [%]	Primer Length [bp]	Amplicon Length [bp]
mNqo1	forward	TGCCATGTACGACAACGGTCC	62,5	60,0	21	145
	reverse	ACGCAGGATGCCACTCTGAA	61,6	55,0	20	
mOpa1 (1)	forward	TCCACCACAGGAAGCTCAAGAAATC	63,0	48,0	25	141
	reverse	TTTTCCCAGCACTCTGATCTCCAAC	63,0	48,0	25	
mRpl4	forward	GTATGGCACTTGGCGGAAGG	61,4	60,0	20	124
	reverse	TGCTCGGAGGGCTCTTTGG	62,0	63,2	19	
mSirt1	forward	ACCTTGGAGCAGGTTGCAGG	62,7	60,0	20	143
	reverse	GGGCACCGAGGAACTACCTGA	63,6	61,9	21	
mTrp53 (l)	forward	GCAGGGCTCACTCCAGCTAC	62,6	65,0	20	124
	reverse	GTGATGGGGACGGGATGCAG	63,0	65,0	20	
mTrp53 (s)	forward	TTCCGGGAGCTGAATGAGGC	62,3	60,0	20	93
	reverse	TCTAGGCTGGAGGCTGGAGTG	63,0	61,9	21	

Gene expression data for *Sod1* and *Ppargc1a* were provided by Dr. Sternberg with his consent. These unpublished data were included to support the interpretation of our findings in conjunction with the expression patterns of the other analyzed genes.

2.2. Methods

2.2.1. Experimental animal model

This experiment was conducted using male C57BL/6NRj mice. After birth, all mice were housed under identical conditions for twelve weeks, maintained at ~ 20 °C with a 12-hour light/dark cycle. During this period, they received a standard nutrient supply before being divided into groups of eight and assigned to one of five specific diets.

Besides Standard diet (SD) the mice were fed with:

- Long chain triglycerides (LCT) diet, ad libitum feeding for 8 weeks
- Long and medium chain triglycerides (LCT/MCT) diet, ad libitum feeding for 8 weeks (70% LCT + 30% MCT)
- Calorie restriction (CR) diet, SD for 6 weeks then feeding 70% once a day repeated for 2 weeks
- Intermittent fasting (IF) diet, SD for 6 weeks then ad libitum feeding for 24h, followed by 24h of fasting repeated for 2 weeks

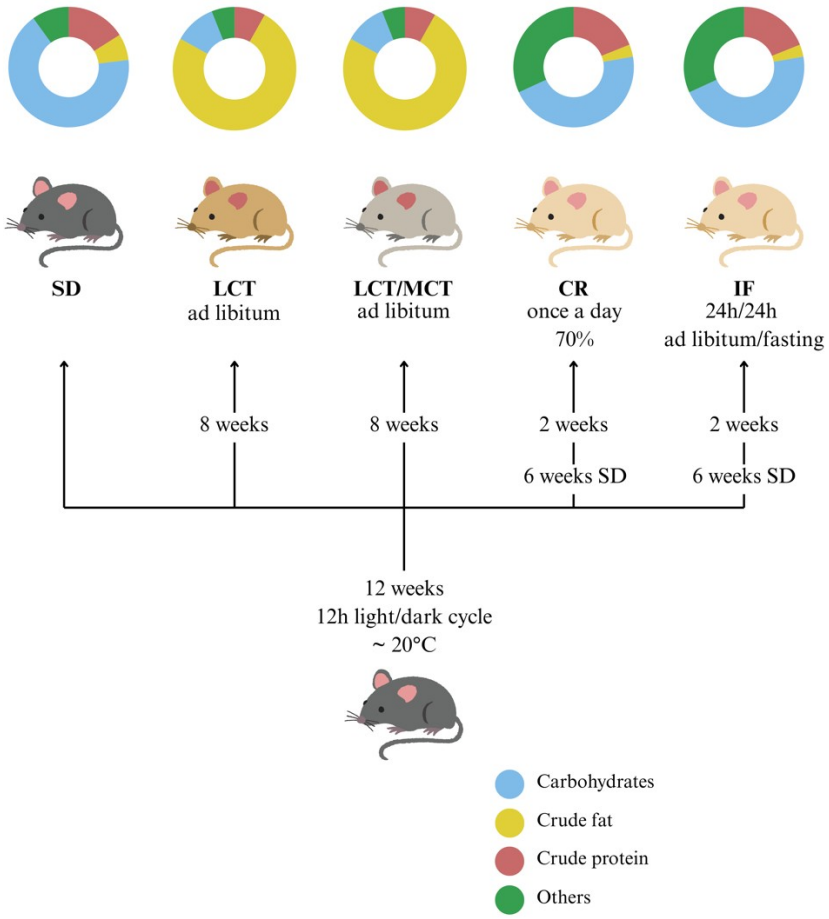

Figure 2.1 **Overview of the dietary interventions applied to the C57BL/6NRj mice**. All mice received the same diet for 12 weeks, after which they were divided into five groups. Besides SD, animals were fed with LCT ad libitum for 8 weeks; LCT/MCT ad libitum for 8 weeks; CR diet with 70% of diet once daily for 2 weeks (6 weeks SD); IF diet with ad libitum access to food for 24 hours followed by 24 hours of fasting repeated for 2 weeks (6 weeks SD). Carbohydrate content includes crude fiber, starch, and sugar. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

The KDs (LCT and LCT/MCT) consisted of approximately 75% fat, whereas the SD was primarily composed of around 65% carbohydrates. The detailed nutritional composition is provided in the supplementary table (Table 5). The LCT diet and the LCT/MCT diet have a fat to carbohydrate ratio of 8:1. Water was provided *ad libitum* across all dietary groups. SD, LCT and LCT/MCT diets were provided *ad libitum*. The CR and IF group were fed the same diet. While the CR group received 70% of the standard diet once daily, the IF group had *ad libitum* access to food for 24 hours, followed by a 24-hour fasting period, repeated in alternating cycles.

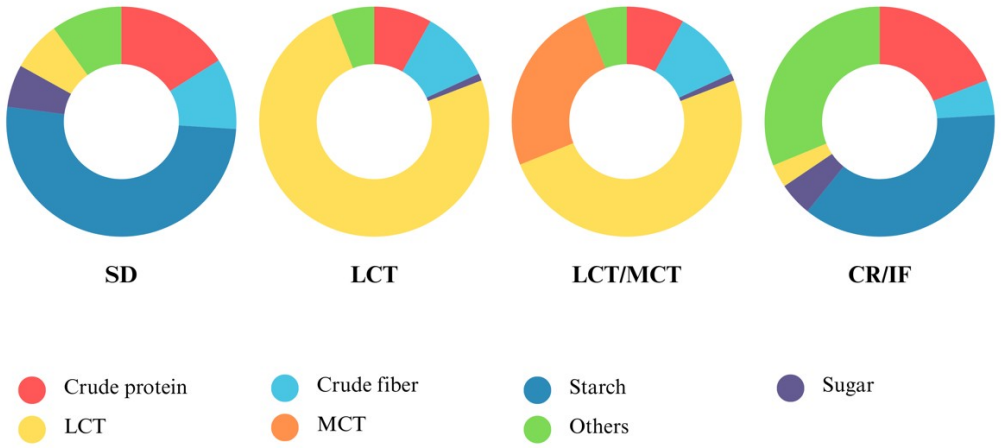

Figure 2.2 **Nutrient composition of the applied diets**. Chart illustrating the nutrient composition (Crude protein, Crude fiber, Starch, Sugar, LCT, MCT, Others) of the diets used in the study. Others includes crude ash, minerals, vitamins and trace elements. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

2.2.2. Sample preparation

All animal procedures were carried out by our collaboration partner Kalina Duszka (University of Vienna) and were authorized by the national authority according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012-TVG 2012 (BMWFW- 66.006/0008-V/3b/2018). For this study hypothalamus and the remaining cortical brain tissue (cortex) were collected. The tissues samples were provided by Dr. Sternberg.

2.2.3. RNA isolation

Total RNA was isolated using guanidine thiocyanate-phenol solution (TriFast), which enables cell lysis and the release of intracellular components. Subsequently, the samples were homogenized to ensure complete disruption of cellular structures. The initial preparation was preprocessed and carried out by Dr. Sternberg. Subsequently, chloroform (Sigma-Aldrich) was added to the homogenates to facilitate phase separation. The incubation and centrifugation lead to three distinct phases: an upper aqueous phase containing RNA, an interphase with DNA, and a lower organic phase comprising proteins and lipids. The RNA-containing aqueous phase was carefully transferred to a new RNase-free tube, taking care to avoid contamination from the interphase and organic phase. To precipitate the RNA, isopropanol (Sigma-Aldrich) and glycogen (Thermo Fisher Scientific) were added to the aqueous phase. Following incubation and centrifugation, the supernatant was carefully removed, and the resulting RNA pellet was

washed with 70% ethanol (Sigma-Aldrich) to eliminate residual impurities. After a final centrifugation step, the ethanol was discarded, and any remaining traces were allowed to evaporate completely, an essential step to prevent ethanol interference in downstream applications. To ensure complete dissolution, the samples were incubated briefly. Finally, the RNA samples were stored at -150°C for long-term preservation.

2.2.4. cDNA synthesis

To prevent RNA degradation, the retrieved mRNA samples were immediately placed on dry ice when taken out, as fluctuations in temperature can adversely affect RNA integrity. Prior to reverse transcription, RNA quantification was performed using the NanoDrop Spectrophotometer (Thermo Fisher Scientific). Before measuring RNA concentrations using the NanoDrop Spectrophotometer, the measurement mode must be set to RNA. Initially, a blank measurement is performed using NF-H_2O to calibrate the device. Subsequently, each RNA sample is applied individually. After each measurement, the measuring pedestal should be carefully wiped clean with a lint-free tissue (Kimberly Clark Professional) to avoid cross-contamination between samples. For this purpose, 1 µl of each RNA sample was applied to the measurement pedestal. The RNA concentration then was determined in ng/µl. The target concentration for downstream applications was approximately 0.5 mg ± 0.3 mg RNA. The reaction mixture was then assembled as follows:

¿

The gDNA wiper mix (Vazyme) eliminates potential genomic DNA (gDNA) contamination. Eliminating gDNA was necessary to avoid non-specific primer binding and ensure the reliability of downstream qPCR measurements. The mixture was then gently pipetted several times to ensure homogeneity and incubated (Liebisch) at 42 °C for 2 minutes. Afterwards, 4 µl of Reverse Transcriptase (Vazyme) was added to the reaction mixture. After thorough mixing, the samples were incubated at 37 °C for 15 minutes to allow reverse transcription, followed by a brief incubation at 85 °C for 5 seconds to inactivate the enzyme. The resulting cDNA was then diluted 1:5 with NF-H_2O and stored in the freezer at -18 °C or directly used for qPCR analysis.

2.2.5. qPCR melting curves and gel electrophoresis

Melting curve analysis was performed to assess the specificity of the amplification. This method ensured that the obtained CT values originated from the desired target sequence. Agarose gel

electrophoresis was used to confirm the expected size of the qPCR products and to further evaluate amplification specificity. PCR products were separated on a 2% agarose gel and visualized under UV light following ethidium bromide staining. The presence of a single band at the expected size indicated successful and specific amplification, while additional bands suggested nonspecific by-products. This step was performed by colleagues prior to the present work. The resulting data were made available and could be included in the subsequent analysis.

2.2.6. Primer design and validation

Primer sequences were designed using the NCBI Primer-BLAST tool (Ye et al., 2012). For this purpose, FASTA sequences of the respective protein-coding genes in *Mus musculus* (taxonomic ID: 10090) were retrieved from the NCBI Nucleotide database. The designed primers were subsequently ordered (Microsynth) and tested for performance. In cases where primers had been previously validated and published in peer-reviewed studies, additional primer efficiency testing was deemed unnecessary. When designing the primers, the maximum amplicon size was restricted to 200 bp to optimize amplification efficiency. Primer melting temperature (Tm) settings were adjusted to a minimum of 60 °C, an optimal value of 63 °C, and a maximum of 66 °C. This deliberate increase in annealing temperature was implemented to minimize the likelihood of non-specific primer binding. Primers were further designed to span exon–exon junctions to avoid amplification of potential gDNA residues. Upon arrival, forward and reverse primers were reconstituted in NF-H_2O to a stock concentration of 100 µM, following the manufacturer's instructions. Subsequently, a 1 µM working solution was prepared for each primer. Therefore, 10 µl of the forward primer and 10 µl of the reverse primer were combined with 980 µl of NF-H_2O, resulting in a final volume of 1 ml.

2.2.7. Polymerase Chain Reaction (PCR)

Previous studies (De Jonge et al., 2007; Liu et al., 2024; Zamani et al., 2020) have demonstrated that hypoxanthine phosphoribosyl transferase (*Hprt*) and ribosomal protein L4 (*Rpl4*) are highly stable housekeeping genes (HKGs). This classification was confirmed by Dr. Sternberg and the genes were therefore selected as HKGs for this study.

384-well plates (Thermo Fisher Scientific) were used for this study. All samples were kept on ice throughout the procedure to prevent enzymatic degradation and maintain integrity. A master mix was prepared, consisting of 1 µl cDNA, 1 µl NF-H_2O and 5 µl ChamQ Universal SYBR

qPCR Master Mix (Vazyme) per well. The total volume of master mix was calculated based on the number of required reactions, including a small excess to compensate for pipetting losses. Each sample was analyzed in duplicate, and a No Template Control (NTC) was performed for each primer pair to detect potential contamination or primer-dimer formation in the absence of cDNA template. 3 µl of the respective primer mix were preloaded into each well of the qPCR plate. Subsequently, 7 µl of the prepared master mix were added to each well. The plate was then sealed with an adhesive film (VWR) and centrifuged (Thermo Fisher Scientific) at 400 rpm for 30 seconds to eliminate air bubbles at the bottom of the wells. RT-qPCR was performed on a QuantStudio 6 Flex Real-Time PCR (Thermo Fisher Scientific) using the cycling protocol shown below (Table 4).

Table 4. PCR cycle temperature

Step	**1**	**2**	**3**	**4**	**5**	**6**
Temperature [°C]	95	62	72	95	60	95
Time [min:sec]	0:10	0:20	0:20	0:15	1:00	0:15

2.2.8. Statistical analysis

Data from qPCR was exported to an excel for further calculations. Gene expression of the targets was analyzed with Graphpad Prism 8. All datasets were initially examined for outliers using Grubbs' test. Gene expression differences were determined using the ΔCt method. Statistical analysis was performed using a one-way ANOVA. For non-parametric data, the Kruskal–Wallis test was applied. Significant differences are represented as follows:

* P ≤ 0,05; ** P ≤ 0,01; *** P ≤ 0,001 for SD.
# P ≤ 0,05; ## P ≤ 0,01; ### P ≤ 0,001 for LCT.
° P ≤ 0,05; °° P ≤ 0,01; °°° P ≤ 0,001 for LCT/MCT.
^ P ≤ 0,05; ^^ P ≤ 0,01; ^^^ P ≤ 0,001 for IF.

Measurements with a standard deviation of CT values greater than 0,3 were repeated to ensure data accuracy. If the standard deviation of the HKGs exceeded 0,3 all other genes were remeasured as well. Otherwise, only the gene with the elevated standard deviation, along with the HKGs, was repeated. If it remained above 0,3 after two repetitions, the corresponding value was excluded from further analysis.

3. Results

3.1. qPCR melting curve and gel electrophoresis

To validate the primers, we conducted a melting curve analysis to determine the ideal annealing temperature and ensure single product formation. A single, sharp peak in the derivative melting curve indicated the presence of a single, specific amplicon, whereas multiple peaks suggested nonspecific products or primer-dimer formation.

The visualization of the melt curve and gel electrophoresis results displays all tested genes, with *Acaca(1)* and *Acaca(2)* described here as a representative example to illustrate the process. As shown in Figure 3.1 below, the melt curve of *Acaca(1)* displays broader peaks with a double peak pattern, indicating potential non-specific amplification or primer-dimer formation, whereas *Acaca(2)* exhibits a single, sharp peak, suggesting specific amplification of the target sequence.

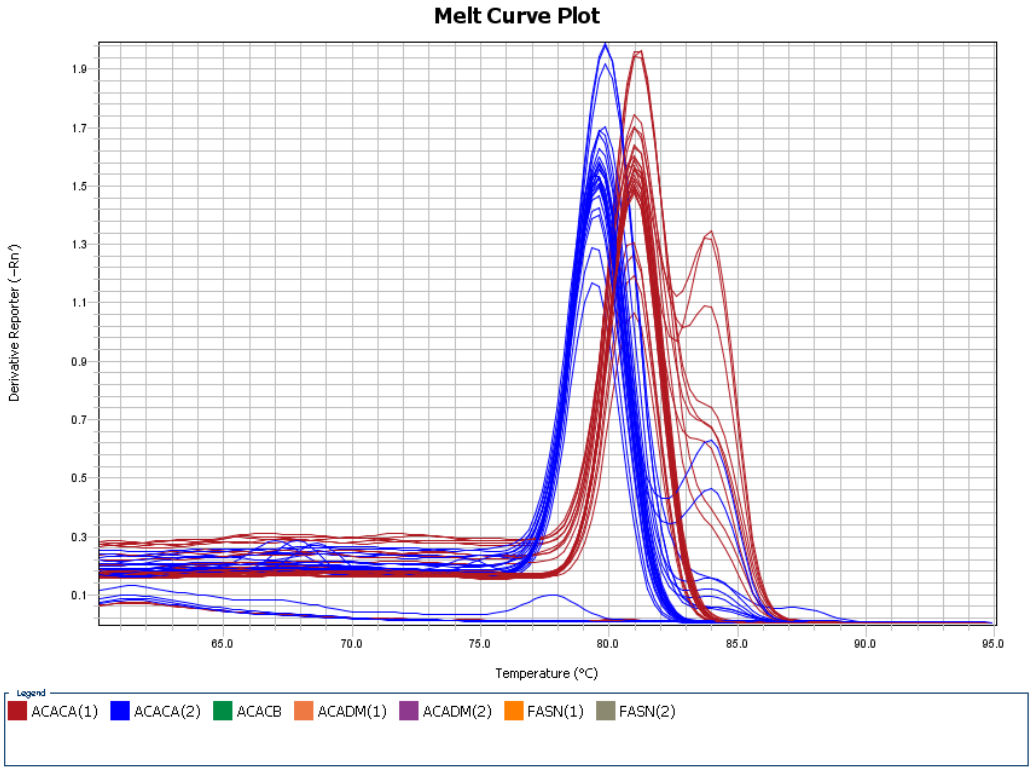

Figure 3.1 **Representative example of a melting curve plot.** Comparison of melt curves for *Acaca(1)* and *Acaca(2)* primers. *Acaca(2)* (in blue) displays a single, sharp peak, indicating a specific and well-defined amplification product. In contrast *Acaca(1)* (in red) shows a broader shape with two distinct peaks, suggesting the presence of non-specific products or suboptimal primer binding.

Afterwards, gel electrophoresis was performed to assess the size and specificity of the PCR products. The separation of DNA fragments by molecular weight allowed for visual confirmation of successful amplification and the absence of nonspecific products.

The gel image below illustrates that amplification with *Acaca(1)* results in two distinct bands, suggesting non-specific amplification or alternative transcript variants, while *Acaca(2)* produces a single, well-defined band, indicating higher primer specificity. Therefore *Acaca(2)* was taken for further qPCR analysis.

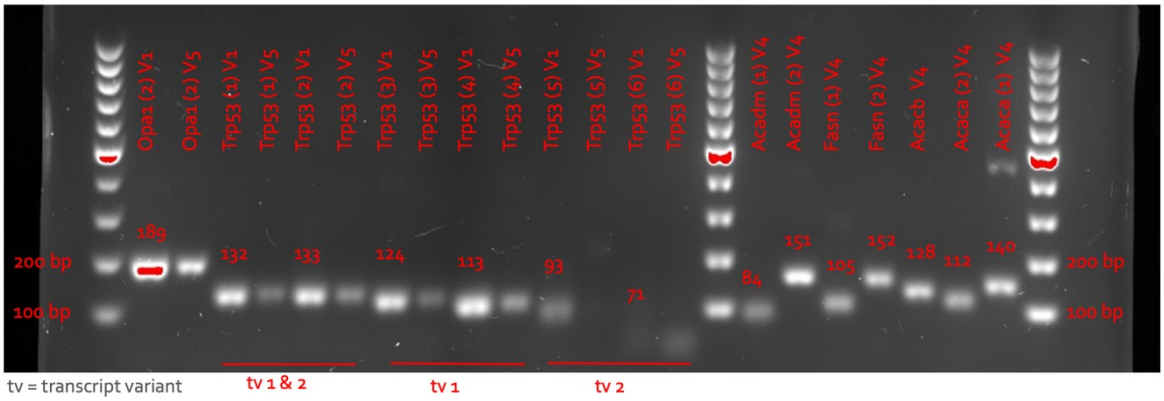

Figure 3.2 **Visualization of qPCR Products on Agarose Gel** of selected primers. Single bands corresponding to expected sizes were observed for all relevant primers.

3.2. Gene expression in the hypothalamus and cortex

Gene expression data from hypothalamic tissue was normalized to the average expression of the HKGs, *Rpl4* and *Hprt*, using the ΔCt method. Therefore, the mean of the two HKGs was used for normalization. For this, the mean HKG Ct value was subtracted from the Ct value of each target gene to obtain ΔCt values. These were subsequently transformed using the 2^-ΔCt method to generate relative expression levels.

For the analyses conducted on cortex tissue, only *Rpl4* was used as a HKG, as it was shown to exhibit greater stability according to laboratory results (data not shown).

3.2.1. Diet effects on hypothalamic lipid gene expression

The results illustrated in Figure 3.3 indicate relatively stable gene expression levels across the different diets. However, *Acaca* expression was significantly decreased in the IF and CR diet groups compared to LCT. The lowest expression was observed in the IF group. In contrast, no statistically significant changes in *Acacb* expression across the dietary groups were detected. *Acadm*, which encodes an enzyme essential for mitochondrial β-oxidation (Koster et al., 2014), was slightly reduced under IF compared to LCT but not to SD. *Crat*, a gene involved in mitochondrial acetyl group transport (Westin et al., 2008), showed a similar expression pattern to *Acadm*, with lower expression levels in the IF group compared to LCT. *Fasn* exhibited high inter-individual variability, particularly in the IF group. Despite this variability, no statistically significant changes were observed. However, expression appeared slightly lower in LCT/MCT compared to SD. The data indicate minimal to no regulation of hypothalamic gene expression related to lipid metabolism across the dietary interventions. None of the diets induced statistically significant changes compared to the standard diet.

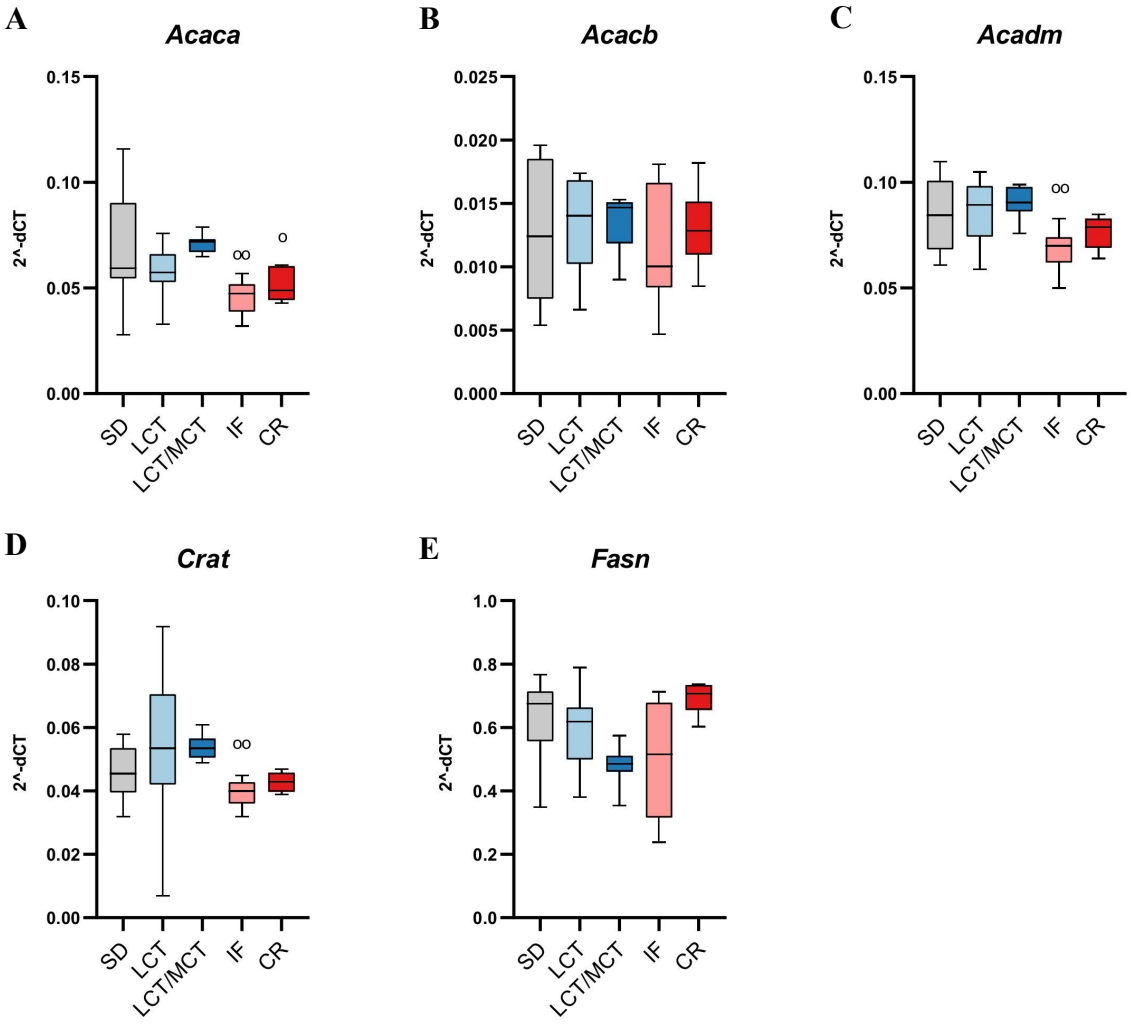

Figure 3.3 **Molecular mRNA expression of lipid metabolism-related genes in SD, LCT, LCT/MCT, CR, and IF in hypothalamic tissue.** Gene expression levels of **(A)** *Acaca,* **(B)** *Acacb,* **(C)** *Acadm,* **(D)** *Crat* and **(E)** *Fasn.* Plots show median, 25th to 75th percentile and minimum to maximum, n = 8 mice per diet group. LCT, LCT/MCT, IF and CR was tested against SD. Statistical analysis was performed using non-parametric Kruskal-Wallis test and one-way ANOVA. Outliers were tested with the Grubb's Test and consequently excluded from the graphical representation. Significant differences are marked with * P ≤ 0,05; ** P ≤ 0,01; *** P ≤ 0,001 for SD; # P ≤ 0,05; ## P ≤ 0,01; ### P ≤ 0,001 for LCT; ° P ≤ 0,05; °° P ≤ 0,01; °°° P ≤ 0,001 for LCT/MCT; ^ P ≤ 0,05; ^^ P ≤ 0,01; ^^^ P ≤ 0,001 for IF. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

3.2.2. Diet effects on hypothalamic mitochondrial dynamics genes

The gene expression data of mitochondrial dynamics-related genes in the hypothalamus (Figure 3.4) reveal both statistically significant differences and non-significant trends across the dietary groups. Notably, *Fis1* expression showed stable levels through different diets, except IF. Although not statistically significant, IF appeared to induce an upward trend in *Fis1* expression. *Mfn1* expression was significantly downregulated in both the CR and IF groups compared to SD. The same significant downregulation was seen in *Opa1* expression. LCT/MCT, IF and CR show significant lower expression than SD. In contrast, *Mfn2* expression did not differ significantly between dietary groups, indicating no detectable diet-induced regulation. Taken together, these findings suggest that mitochondrial dynamics-related gene expression in the hypothalamus might be influenced by the dietary interventions examined. These differences were observed and will be further discussed.

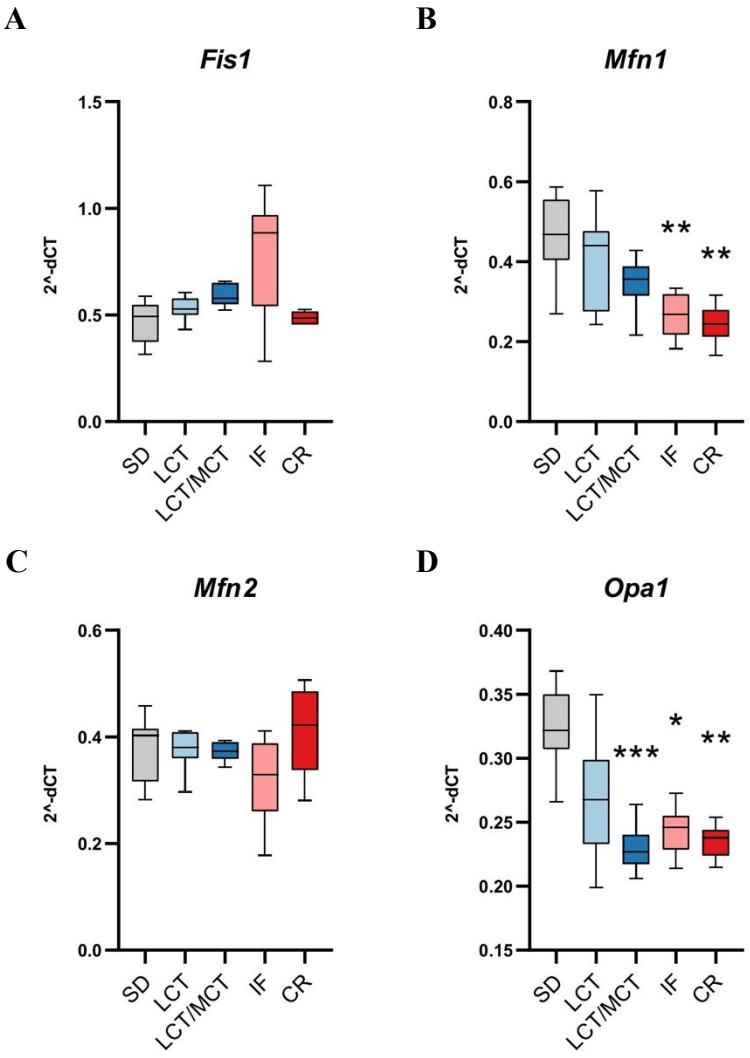

Figure 3.4 **Molecular mRNA expression of genes related to mitochondrial dynamics in SD, LCT, LCT/MCT, CR, and IF in hypothalamic tissue.** Gene expression levels of **(A)** *Fis1,* **(B)** *Mfn1,* **(C)** *Mfn2* and **(D)** *Opa1.* Plots show median, 25th to 75th percentile and minimum to maximum, n = 8 mice per diet group. LCT, LCT/MCT, IF and CR was tested against SD. Statistical analysis was performed using non-parametric Kruskal-Wallis test and one-way ANOVA. Outliers were teste with the Grubb's test and consequently excluded from the graphical representation. Significant differences are marked with * P ≤ 0,05; ** P ≤ 0,01; *** P ≤ 0,001 for SD; # P ≤ 0,05; ## P ≤ 0,01; ### P ≤ 0,001 for LCT; ° P ≤ 0,05; °° P ≤ 0,01; °°° P ≤ 0,001 for LCT/MCT; ^ P ≤ 0,05; ^^ P ≤ 0,01; ^^^ P ≤ 0,001 for IF. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

3.2.3. Diet effects on hypothalamic *Trp53* expression

The results illustrated in Figure 3.5 indicate that *Trp53(l)* expression reveal a significant downregulation in the LCT dietary group compared to the SD. Conversely, the CR group exhibits a significant upregulation relative to the LCT group. Notably, the LCT and IF groups display larger interquartile ranges, indicating greater variability in gene expression levels within these groups. In contrast, *Trp53(s)* shows no statistically significant differences in expression between the dietary interventions. However, the LCT and CR groups are characterized by higher interquartile ranges, suggesting increased dispersion of gene expression values in these groups. Overall, expression levels of the *Trp53(s)* are observed in a substantially lower range (approximately 0.0001 to 0.0004) compared to *Trp53(l),* which generally exhibits higher expression values around 0.1 to 0.2. In contrast to both *Trp53* isoforms, significant differences were observed in the expression of *Ppargc1a*. Both the IF and CR diet groups showed a significant downregulation of *Ppargc1a* compared to the SD group. However, the LCT and LCT/MCT groups showed no change in gene expression.

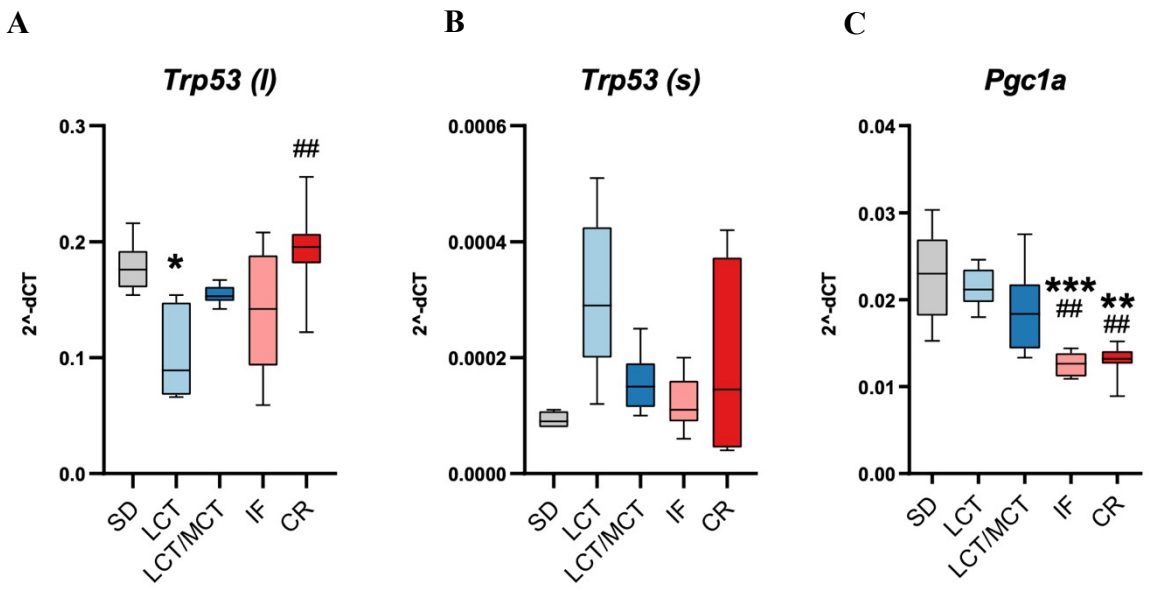

Figure 3.5 **Molecular mRNA expression of short and long isoform of *Trp53* and *Ppargc1a* in SD, LCT, LCT/MCT, CR, and IF in hypothalamic tissue.** Gene expression levels of **(A)** *Trp53(l),* **(B)** *Trp53(s)* and **(C)** *Ppargc1a* (labelled as Pgc1a in the figure). Plots show median, 25th to 75th percentile and minimum to maximum, n = 4-8 mice per diet group. LCT, LCT/MCT, IF and CR was tested against SD. Statistical analysis was performed using non-parametric Kruskal-Wallis test and one-way ANOVA. Outliers were tested with the Grubb's test and consequently excluded from the graphical representation. Significant differences are marked with * P ≤ 0,05; ** P ≤ 0,01; *** P ≤ 0,001 for SD; # P ≤ 0,05; ## P ≤ 0,01; ### P ≤ 0,001 for LCT; ° P ≤ 0,05; °° P ≤ 0,01; °°° P ≤ 0,001 for LCT/MCT; ^ P ≤ 0,05; ^^ P ≤ 0,01; ^^^ P ≤ 0,001 for IF. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

3.2.4. Diet effects on cortical NRF2-regulated antioxidant genes

The expression levels *Sod1* and *Gpx1* followed a similar trend. While gene expression levels in the SD, LCT, and CR groups were largely comparable and showed no significant variations, notable changes were observed in response to the LCT/MCT and IF diet. Specifically, both the LCT/MCT and IF diets induced a significant upregulation of *Sod1* and *Gpx1* gene expression. Expression levels were significantly elevated relative to those in the SD and LCT groups, which may indicate a potential diet-associated modulation of antioxidant defense-related gene expression under these conditions.

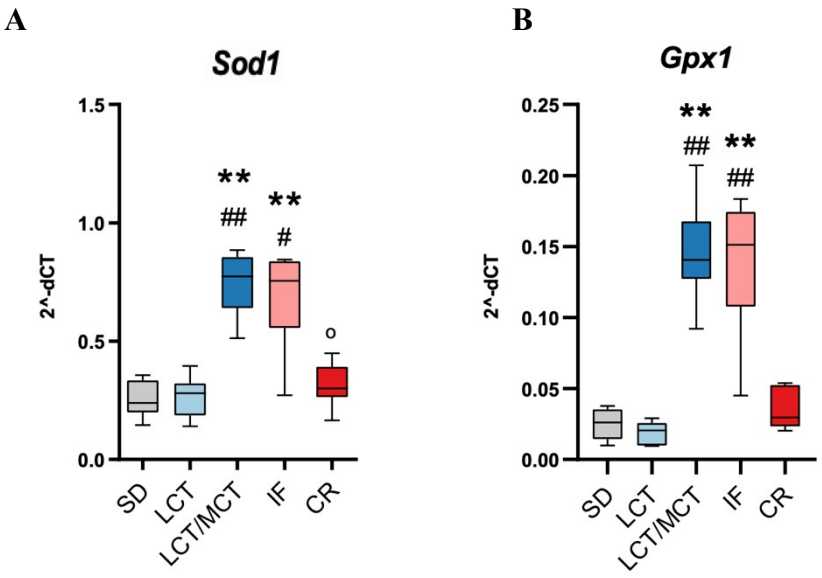

Figure 3.6 **Molecular mRNA expression of classical antioxidant enzymes regulated by NRF2 in SD, LCT, LCT/MCT, CR, and IF in cortical tissue.** Gene expression levels of **(A)** *Sod1* and **(B)** *Gpx1*. Plots show median, 25th to 75th percentile and minimum to maximum, n = 6-8 mice per diet group. LCT, LCT/MCT, IF and CR was tested against SD. Statistical analysis was performed using non-parametric Kruskal-Wallis test and one-way ANOVA. Outliers were tested with the Grubb's test and consequently excluded from the graphical representation. Significant differences are marked with * P ≤ 0,05; ** P ≤ 0,01; *** P ≤ 0,001 for SD; # P ≤ 0,05; ## P ≤ 0,01; ### P ≤ 0,001 for LCT; ° P ≤ 0,05; °° P ≤ 0,01; °°° P ≤ 0,001 for LCT/MCT; ^ P ≤ 0,05; ^^ P ≤ 0,01; ^^^ P ≤ 0,001 for IF. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

3.2.5. Diet effects on cortical NRF2-linked detox and redox genes

For *Sirt1*, significant higher median expression levels were observed in the IF group compared to SD. An increasing trend in expression appears to be present in the LCT/MCT and CR groups, although no statistically significant differences were observed. Statistically significant differences were detected between IF vs. SD and IF vs. LCT. *Nqo1* expression exhibited a similar pattern. The IF group displayed significant elevated median values compared to SD and LCT. No statistically significant differences in gene expression were observed for the other diets compared to SD. In both genes, inter-individual variability was observed across all groups. Like the other two genes, *Ppargc1a* showed a significant upregulation in the IF group compared to the SD group. Interestingly, the LCT/MCT group also exhibited a significant upregulation. All other diets showed no significant changes in gene expression.

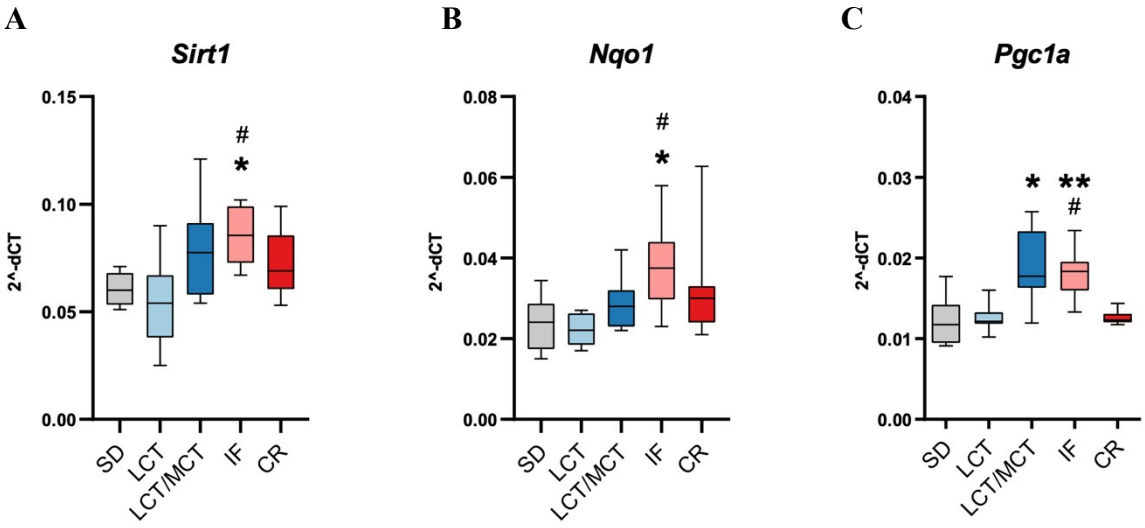

Figure 3.7 **Molecular mRNA expression of NRF2-associated detox and redox genes in SD, LCT, LCT/MCT, CR, and IF in cortical tissue.** Gene expression levels of **(A)** *Sirt1,* **(B)** *Nqo1* and **(C)** *Ppargc1a* (labelled as Pgc1a in the figure). Plots show median, 25th to 75th percentile and minimum to maximum, n = 6-8 mice per diet group. LCT, LCT/MCT, IF and CR was tested against SD. Statistical analysis was performed using non-parametric Kruskal-Wallis test and one-way ANOVA. Outliers were tested with the Grubb's test and consequently excluded from the graphical representation. Significant differences are marked with * P ≤ 0,05; ** P ≤ 0,01; *** P ≤ 0,001 for SD; # P ≤ 0,05; ## P ≤ 0,01; ### P ≤ 0,001 for LCT; ° P ≤ 0,05; °° P ≤ 0,01; °°° P ≤ 0,001 for LCT/MCT; ^ P ≤ 0,05; ^^ P ≤ 0,01; ^^^ P ≤ 0,001 for IF. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

4. Discussion

4.1. MCT induced changes in hypothalamic gene expression

We investigated the effects of ketogenic diets and energy restriction paradigms on hypothalamic gene expression in mice. A particular focus was given to genes involved in lipid metabolism, mitochondrial dynamics, and cellular stress responses. Based on previous findings (Sternberg et al., unpublished data) of cellular adaptations to MCTs in LCT-based KDs, we hypothesized that increased levels of C8 and C10 fatty acids (MCTs) would trigger transcriptional changes in nutrient sensing and metabolic pathways.

Due to the high fat content of the LCT and LCT/MCT diets (~75%), we assumed that genes directly involved in lipid metabolism (*Acaca, Acacb, Acadm, Crat, Fasn*) would show increased expression in these diet groups. However, the data did not reveal a significant upregulation of lipid-related genes in the hypothalamus under these ketogenic conditions (Figure 3.3). Neither the LCT-KD nor the combined LCT/MCT-KD induced significant changes in the expression of lipid metabolism related genes in the hypothalamus. One possible explanation for this observation is that transcriptional changes predominantly take place in peripheral tissues, like the liver, where dietary fats are actively processed, and which are more directly involved in lipid metabolism. Previous studies support our assumptions (Kennedy et al., 2007). Notably, depending on the metabolic function of the gene, both up- and downregulation can occur. Nasser et al. (2022) showed that ketogenic diets downregulate lipogenic enzymes such as *Fasn* and *Acc* in the liver, reflecting a metabolic shift away from lipogenesis toward fatty acid oxidation and ketone body production (Nasser et al., 2022). Consequently, the hypothalamic response may be more subtle or secondary, resulting in less pronounced changes in gene expression compared to metabolically active organs.

To date, no studies so far have demonstrated alterations in hypothalamic gene expression related to lipid metabolism in response to a KD. However, some studies have reported region-specific metabolic alterations in other brain areas. Pathak et al. (2022) demonstrated that a KD in middle aged female mice enhanced cognitive performance and region-specific metabolic profiles in the cortex and hippocampus (Pathak et al., 2022). Roslund et al. (2024) further demonstrated that a KD in the same mouse model induced tissue- and region-specific metabolic shifts, including increased ketone bodies (Roslund et al., 2024). These alterations likely reflect shifts in energy

metabolism and increased utilization of lipid-derived ketone bodies, suggesting an indirect involvement of cerebral lipid metabolism.

Similarly, metabolic interventions, IF and CR, did not significantly alter the expression of genes involved in lipid metabolism in the hypothalamus. However, some genes associated with mitochondrial fusion (*Mfn1* and *Opa1*) showed significantly downregulated expression under the energy restriction conditions (Figure 3.4). Comparable expression patterns were observed for *Ppargc1a* (Figure 3.5). The demonstrated downregulation of *Ppargc1a* in the IF and CR diet group, may reflect an adaptive response to energy scarcity, characterized by a reduction in mitochondrial biogenesis and oxidative capacity. This may serve to conserve energy and limit ROS production in hypothalamic neurons. The concurrent downregulation of *Ppargc1a* and the mitochondrial fusion genes *Mfn1* and *Opa1* observed, suggests a regulatory relationship, in which reduced *Ppargc1a* expression may directly contribute to the decreased expression of fusion-related genes, as described in Wang et al. (2022) (J. Wang et al., 2022). Controversially, previous studies have reported no consistent changes in the expression of mitochondrial fusion markers *Mfn1, Mfn2, Opa1* and the biogenesis factor *Ppargc1a* under conditions of energy restriction. For instance, Mehrabani et al. (2020) reviewed studies across various tissues and found that expression of these markers often remained unchanged (Mehrabani et al., 2020). Notably, Nobis et al. (2018) specifically investigated in the hypothalamus and also reported unaltered protein levels of Mfn1, Mfn2, Opa1 and Ppargc1*a* in rats subjected to activity-based anorexia (Nobis et al., 2018). In contrast, increased mitochondrial fission appears to be a more consistent response. Nobis et al. (2018) observed a significant increase in phosphorylated DRP1 in the hypothalamus, suggesting enhanced DRP1 activation. Similarly, Zhao et al. (2018) reported increased DRP1 gene and protein expression under caloric restriction, particularly in cardiac tissue (Zhao et al., 2018). Taken together, these observations point toward a possible role of mitochondrial fission in the adaptive response to energy restriction, while changes in fusion and biogenesis remain less evident.

In addition, *Trp53(l)*, a gene involved in stress signaling and apoptosis, showed a significant downregulation in expression in the LCT group (Figure 3.5) but no alterations were observed in any of the other dietary groups. To date, no research directly addresses how LCT-KD affect Trp53 gene expression in the hypothalamus. While *Trp53* is typically involved in cellular stress responses and apoptosis, the mechanisms underlying this specific induction remain unclear. One possible explanation could be a metabolic or oxidative stress response brought on by the

hypothalamus' LCFA metabolism. However, the lack of comparable impact in the combined LCT/MCT-KD group raises the possibility that MCT could reduce or even reverse this reaction. To clarify the underlying mechanisms, more research is necessary.
In contrast, Wei et al. (2024) demonstrated that long term KDs induce *p53* mediated cellular senescence in peripheral tissues such as liver, heart and kidney (Wei et al., 2024). However, the study did not report any changes in *p53* expression in the hypothalamus.

Although, we initially expected transcriptional changes in genes involved in lipid metabolism due to the high LCT and MCT content of the diets, our data did not reveal significant alterations in these genes within the hypothalamus. In contrast, the observed downregulation of *Mfn1*, *Opa1* and *Ppargc1a* under IF and CR could indicate an adaptive response aimed at conserving energy and limiting oxidative stress in hypothalamic neurons. Additionally, Trp53 expression was reduced under LCT feeding, potentially reflecting altered cellular stress responses. Taken together, these findings may point to hypothalamus-specific transcriptional adaptations to metabolic interventions, particularly concerning mitochondrial function. However, further research is required to validate and extend these observations.

One limitation of our study is that only male C57BL/6NRj mice were included, which does not account for potential sex-specific differences in hypothalamic gene expression. In addition, the conclusions are based solely on mRNA expression levels. Therefore, future studies should include complementary approaches such as Western blotting or immunohistochemistry to assess protein expression.

4.2. NRF2 dependent antioxidant response in the cortex

In recent years, research on the NRF2 pathway has gained significant interest due to its role in cellular defense and aging-related processes. As previously shown by Yang et al. (2024) the NRF2 antioxidant pathway represents a promising target for geroprotection (Y. Yang et al., 2024). Despite these findings, gene expression analysis alone is insufficient to establish causal relationships, and further investigations are warranted. One limitation of this study lies in the complex regulation of NRF2, which is influenced by multiple factors. Nonetheless, several notable NRF2 downstream targets, including *Sod1, Nqo1*, and *Gpx1*, emerged as of particular interest. Although our study was conducted in mice, relevant findings from rat studies were also considered to support the interpretation of the results.

Sod1 and *Gpx1* expression levels were elevated in the LCT/MCT and IF groups (Figure 3.6). A similar upregulation was observed in *Ppargc1a* under the same dietary conditions (Figure 3.7). Notably, *Nqo1* and *Sirt1* also showed increased expression in response to the IF diet, but not under any KD (Figure 3.7). As shown in Mehrabani et al. (2020) fasting has been associated with activation of the NRF2 signaling pathway, leading to the induction of cytoprotective and antioxidant gene expression (Mehrabani et al., 2020). This observation may be explained by the fact that both IF and KDs have been suggested to induce mild oxidative stress (Milder et al., 2010). As a compensatory mechanism, this could activate the NRF2 signaling pathway, leading to increased transcription of downstream targets such as *Sod1*, *Gpx1*, and *Nqo1*, which help restore redox homeostasis and prevent potential damage caused by excessive ROS. This assumption is supported by prior studies (L. Li et al., 2013; Milder et al., 2010).

The observed upregulation of NRF2-regulated antioxidant genes such as *Sod1*, *Gpx1*, and *Nqo1* may reflect a mitohormetic response, suggesting that mild metabolic stress from interventions triggers protective transcriptional responses without leading to measurable oxidative damage (Calabrese et al., 2012; Hine, 2012; Holmström et al., 2016). In this context, transient increases in mitochondrial ROS could act as signaling molecules that trigger activation of the NRF2 pathway, resulting in enhanced expression of cellular defense genes. Interestingly, *Sod1* and *Gpx1* expression levels were increased in both the LCT/MCT and IF diet groups, while *Nqo1* upregulation was observed only in the IF group.

Interestingly, the LCT/MCT-KD, but not the LCT-KD, induced increased expression of *Sod1* and *Gpx1* in the cortex. This difference may be attributed to the specific metabolic properties of MCTs, which are more rapidly metabolized into ketone bodies and cross the blood-brain barrier more efficiently (Dunn et al., 2023). Enhanced mitochondrial oxidation and increased ROS signaling under MCT influence could trigger an adaptive antioxidant response via the NRF2 pathway. Furthermore, MCTs may support NAD^+-dependent activation of SIRT1, which in turn promotes mitochondrial biogenesis and antioxidant gene expression through PGC-1α and NRF2. These findings suggest that the presence of MCTs in KD may hold a central role in shaping cortical redox homeostasis.

The upregulation of *Ppargc1a* in the LCT/MCT and IF groups may reflect an adaptive mitochondrial response to metabolic interventions (Figure 3.7). PGC-1α is the key regulator of

mitochondrial biogenesis and has been shown to interact functionally with NRF2 signaling (Gureev et al., 2019). In this context, increased *Ppargc1a* expression in the mice cortex could support mitochondrial maintenance and contribute to enhanced antioxidant defense by promoting NRF2 activity and its downstream targets. This is in line with previous studies (Aquilano et al., 2013) demonstrating that PGC-1α and NRF2 act synergistically to protect against oxidative stress and sustain cellular energy homeostasis, particularly in the brain. Notably, *Sirt1* expression was also elevated in the IF intervention. SIRT1, a NAD^+-dependent deacetylase that plays a role in cellular stress responses (Gillum et al., 2011), has been shown to modulate both PGC-1α and NRF2 activity. Through deacetylation, SIRT1 can activate PGC-1α and enhance NRF2 stability and transcriptional activity, thereby promoting the expression of antioxidant genes (Ding et al., 2016).

Thus, the concurrent upregulation of *Ppargc1a*, *Sirt1*, and NRF2-associated genes such as *Sod1*, *Gpx1*, and *Nqo1* could indicate a coordinated transcriptional response to dietary interventions, potentially reflecting a mitohormetic adaptation aimed at supporting redox balance and mitochondrial function in the cortex. Taken together, the findings suggest that dietary interventions such as IF and KDs may activate the NRF2 pathway and related antioxidant genes in the mouse cortex. This transcriptional response likely reflects an adaptive mechanism involving mitohormesis and mitochondrial biogenesis, as indicated by the concurrent upregulation of *Ppargc1a* and *Sirt1*. These results highlight the potential of metabolic modulation to enhance redox resilience in the brain.

This study focused exclusively on the mouse cortex, which limits conclusions about region-specific effects in the brain. Future studies should include additional areas such as the hippocampus, hypothalamus or cerebellum to better capture differential responses. Moreover, only mRNA expression was assessed. As mRNA levels do not necessarily reflect protein abundance or activity, complementary analyses, like Western blotting or enzyme assays, are needed to validate the functional impact. Additionally, potential sex-specific responses were not addressed in this study. Including both male and female animals in future experiments could help clarify whether the observed effects are sex-dependent. Finally, further research is required to establish causal relationships between dietary interventions, NRF2 pathway activation, and functional outcomes.

5. Summary

This masterproject investigated the effects of various dietary interventions on hypothalamic gene expression in male C57BL/6NRj mice, with a focus on lipid metabolism, mitochondrial dynamics, and cellular stress responses. Animals were classified to either standard diet (SD), a long-chain triglyceride based ketogenic diet (LCT), a combined long- and medium-chain triglyceride based ketogenic diet (LCT/MCT), intermittent fasting (IF) or caloric restriction (CR). Gene expression analyses from hypothalamus tissue was run via RT-qPCR. Contrary to our initial hypothesis, ketogenic diets (KDs) did not significantly alter the expression of genes involved in lipid metabolism in the mouse hypothalamus. These findings suggest that transcriptional regulation of lipid processing genes in response to KDs may predominantly occur in peripheral metabolic tissues such as the liver. In contrast, fasting-related dietary conditions (IF and CR) induced significant downregulation of genes associated with mitochondrial fusion and biogenesis (*Mfn1*, *Opa1*, and *Ppargc1a*). This suggests a hypothalamic adaptation to energy scarcity by limiting mitochondrial activity and potential oxidative stress.

Overall, our results point to specific transcriptional adaptations in the mouse hypothalamus in response to metabolic interventions, particularly under fasting conditions. Further studies are required to confirm these preliminary findings and to enable more precise conclusions about the role of hypothalamic gene expression in response to LCT- and LCT/MCT-KD as well as IF and CR.

The second part of the thesis aimed to investigate whether the same dietary interventions modulate antioxidant defense mechanisms in the mouse cortex, with a particular focus on the NRF2 signaling pathway and its downstream targets. The results suggest that certain genes, such as *Sod1* and *Gpx1*, exhibited increased expression in both the LCT/MCT and IF groups, whereas *Nqo1* was upregulated primarily in response to IF. This differential gene regulation may indicate a context-dependent transcriptional activation of NRF2-associated targets. These findings could reflect an adaptive response to mild metabolic stress, potentially mediated by mitohormetic mechanisms. The concurrent upregulation of the indirect regulators *Ppargc1a* and *Sirt1* in specific intervention groups may further suggest a role for mitochondrial adaptations in supporting redox homeostasis. However, it is important to note that mRNA levels alone do not necessarily translate into changes in protein abundance or enzymatic activity. As such, the functional implications of these transcriptional patterns remain speculative.

Taken together, the results provide preliminary indications that dietary interventions such as IF and MCT supplementation may modulate antioxidant gene expression in the mice cortex. To evaluate the physiological relevance of these changes, further studies will be required.

6. Zusammenfassung

Diese Masterarbeit untersuchte die Auswirkungen verschiedener diätetischer Interventionen auf die Genexpression im Hypothalamus männlicher C57BL/6NRj-Mäuse mit einem Schwerpunkt auf Lipidstoffwechsel, mitochondrialer Dynamik und zellulärer Stressantwort im Hypothalamus. Die Tiere erhielten entweder Standardfutter (SD), eine auf langkettige Fettsäuren basierende ketogene Diät (LCT), eine auf kombinierte lang- und mittelkettige Fettsäuren basierende ketogene Diät (LCT/MCT), intermittierendes Fasten (IF) oder Kalorienrestriktion (CR). Die Genexpressionsanalysen wurden mittels RT-qPCR durchgeführt. Entgegen unserer ursprünglichen Hypothese führten ketogene Diäten (KDs) nicht zu einer signifikanten Veränderung der Expression der Gene im Hypothalamus, die am Lipidstoffwechsel beteiligt sind. Diese Ergebnisse deuten darauf hin, dass die transkriptionelle Regulation von lipidverarbeitenden Genen als Reaktion auf KDs vorwiegend in peripheren Stoffwechselgeweben, wie der Leber, stattfinden könnte. Im Gegensatz dazu führten energiereduzierte Diäten (IF und CR) zu einer signifikanten Abnahme der Expression von Genen, die mit mitochondrialer Fusion und Biogenese assoziiert sind (*Mfn1*, *Opa1* und *Ppargc1a*). Dies könnte auf eine hypothalamische Anpassung an den Energiemangel hinweisen, bei der mitochondriale Aktivität und potenzieller oxidativer Stress reduziert werden.
Insgesamt sprechen die Ergebnisse für spezifische transkriptionelle Anpassungen im Hypothalamus als Reaktion auf metabolische Interventionen, vor allem im Kontext des Fastens. Weitere Studien sind notwendig, um diese ersten Hinweise zu bestätigen und genauere Aussagen über die Rolle der hypothalamischen Genexpression in Reaktion auf LCT- und LCT/MCT-KDs sowie energieeinschränkende Interventionen, wie IF und CR zu ermöglichen.

Der zweite Teil der Arbeit zielte darauf ab, zu untersuchen, ob dieselben diätetischen Interventionen antioxidative Abwehrmechanismen im Kortex der Mäuse modulieren, mit besonderem Fokus auf den NRF2-Signalweg und dessen Zielgene. Die Ergebnisse deuten darauf hin, dass bestimmte Gene wie *Sod1* und *Gpx1* sowohl bei der LCT/MCT- als auch bei der IF-Diät eine erhöhte Expression aufwiesen, während *Nqo1* hauptsächlich in Reaktion auf IF hochreguliert wurde. Diese differenzielle Genregulation könnte auf eine selektive oder kontextabhängige Aktivierung NRF2-assoziierter Zielgene auf transkriptioneller Ebene hinweisen. Die beobachteten Veränderungen könnten eine adaptive Antwort auf milden metabolischen Stress widerspiegeln, möglicherweise vermittelt durch mitohormetische Mechanismen. Die gleichzeitige Hochregulation der Regulatoren *Ppargc1a* und *Sirt1* in bestimmten Interventionsgruppen könnte zusätzlich auf mitochondriale Anpassungen zur

Unterstützung der Redox-Homöostase hindeuten. Es ist jedoch zu beachten, dass mRNA-Level nicht zwangsläufig Rückschlüsse auf enzymatische Aktivität zulassen. Entsprechend bleiben die funktionellen Implikationen dieser transkriptionellen Veränderungen spekulativ.

Zusammenfassend lieferten die Ergebnisse erste Hinweise darauf, dass diätetische Interventionen wie IF und MCT-Supplementierung die Expression von Genen der antioxidativen Abwehr im Kortex der Mäuse modulieren könnten. Zur Bewertung der physiologischen Relevanz dieser Veränderungen sind weiterführende Studien erforderlich.

7. Abbreviations

ACAD	Acetyl-CoA dehydrogenases
ACC (1/2)	Acetyl-CoA carboxylase 1/2
ALS	Amyotrophic lateral sclerosis
AMPK	Adenosine 5 'monophosphate-activated protein kinase
ARE	Antioxidant response element
CACT	Carnitine/acetylcarnitine translocase
CPT1	Carnitine palmitoyltransferase 1
CR	Caloric restriction
CRAT	Carnitine O-acetyltransferase
CROT	Carnitine octanyltransferase
DHA	Docosahexaenoic acid
DRP1	Dynamin-related protein 1
ER	Endoplasmatic reticulum
FASN	Fatty acid synthase
FIS1	Mitochondrial fission 1 protein
gDNA	Genomic DNA
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
HKG	House keeping gene
IF	Intermittent fasting
IMM	Inner mitochondrial membrane
KB	Ketone bodies
KD	Ketogenic diet
KEAP1	Kelch-like ECH-associated protein 1
LCT	Long-chain triglyceride
Maf	Musculoaponeurotic fibrosarcoma
MCAD	Medium-chain acyl-CoA dehydrogenase
MCFA	Medium-chain fatty acids
MCT	Medium-chain triglyceride
MDM2	Murine double minute 2

MFF	Mitochondrial fission factor
MFN	Mitofusin protein
MFN1/2	Mitofusin-1/2
NDs	Neurodegenerative diseases
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
Nqo1	NAD(P)H:quinone acceptor oxidoreductase 1
NRF2	Nuclear factor erythroid-derived 2-like 2
$O_2^{\bullet-}$	Superoxide radicals
OMM	Outer mitochondrial membrane
OPA1	Optic atrophy 1
PGC-1α	Peroxisome proliferator-activated receptor gamma-coactivator-1 α
ROS	Reactive oxygen species
SD	Standard diet
Sirt1	Silencing information regulator 2 related enzyme 1
SOD	Superoxide dismutase
Tm	Melting temperature

8. Figures and Tables

8.1. Figures

25th to 75th percentile and minimum to maximum, n = 8 mice per diet group. LCT, LCT/MCT, IF and CR was tested against SD. Statistical analysis was performed using non-parametric Kruskal-Wallis test and one-way ANOVA. Outliers were teste with the Grubb's test and consequently excluded from the graphical representation. Significant differences are marked with * P ≤ 0,05; ** P ≤ 0,01; *** P ≤ 0,001 for SD; # P ≤ 0,05; ## P ≤ 0,01; ### P ≤ 0,001 for LCT; ° P ≤ 0,05; °° P ≤ 0,01; °°° P ≤ 0,001 for LCT/MCT; ^ P ≤ 0,05; ^^ P ≤ 0,01; ^^^ P ≤ 0,001 for IF. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

Figure 3.5 Molecular mRNA expression of short and long isoform of *Trp53* and *Ppargc1a* in SD, LCT, LCT/MCT, CR, and IF in hypothalamic tissue. Gene expression levels of (A) *Trp53(l),* (B) *Trp53(s)* and (C) *Ppargc1a* (labelled as Pgc1a in the figure). Plots show median, 25th to 75th percentile and minimum to maximum, n = 4-8 mice per diet group. LCT, LCT/MCT, IF and CR was tested against SD. Statistical analysis was performed using non-parametric Kruskal-Wallis test and one-way ANOVA. Outliers were tested with the Grubb's test and consequently excluded from the graphical representation. Significant differences are marked with * P ≤ 0,05; ** P ≤ 0,01; *** P ≤ 0,001 for SD; # P ≤ 0,05; ## P ≤ 0,01; ### P ≤ 0,001 for LCT; ° P ≤ 0,05; °° P ≤ 0,01; °°° P ≤ 0,001 for LCT/MCT; ^ P ≤ 0,05; ^^ P ≤ 0,01; ^^^ P ≤ 0,001 for IF. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

Figure 3.6 Molecular mRNA expression of classical antioxidant enzymes regulated by NRF2 in SD, LCT, LCT/MCT, CR, and IF in cortical tissue. Gene expression levels of (A) *Sod1* and (B) *Gpx1*. Plots show median, 25th to 75th percentile and minimum to maximum, n = 6-8 mice per diet group. LCT, LCT/MCT, IF and CR was tested against SD. Statistical analysis was performed using non-parametric Kruskal-Wallis test and one-way ANOVA. Outliers were tested with the Grubb's test and consequently excluded from the graphical representation. Significant differences are marked with * P ≤ 0,05; ** P ≤ 0,01; *** P ≤ 0,001 for SD; # P ≤ 0,05; ## P ≤ 0,01; ### P ≤

0,001 for LCT; ° P ≤ 0,05; °° P ≤ 0,01; °°° P ≤ 0,001 for LCT/MCT; ^ P ≤ 0,05; ^^ P ≤ 0,01; ^^^ P ≤ 0,001 for IF. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

Figure 3.7 Molecular mRNA expression of NRF2-associated detox and redox genes in SD, LCT, LCT/MCT, CR, and IF in cortical tissue. Gene expression levels of (A) *Sirt1*, (B) *Nqo1* and (C) *Ppargc1a* (labelled as Pgc1a in the figure). Plots show median, 25th to 75th percentile and minimum to maximum, n = 6-8 mice per diet group. LCT, LCT/MCT, IF and CR was tested against SD. Statistical analysis was performed using non-parametric Kruskal-Wallis test and one-way ANOVA. Outliers were tested with the Grubb's test and consequently excluded from the graphical representation. Significant differences are marked with * P ≤ 0,05; ** P ≤ 0,01; *** P ≤ 0,001 for SD; # P ≤ 0,05; ## P ≤ 0,01; ### P ≤ 0,001 for LCT; ° P ≤ 0,05; °° P ≤ 0,01; °°° P ≤ 0,001 for LCT/MCT; ^ P ≤ 0,05; ^^ P ≤ 0,01; ^^^ P ≤ 0,001 for IF. SD – standard diet, LCT – long chain triglycerides, LCT/MCT – long and medium chain triglycerides, IF – intermittent fasting, CR – caloric restriction.

8.2. Tables

8.3. Supplementary table

Table 5. Nutrient composition

	SD	LCT	LCT/MCT	CR	IF
Company	ssniff	ssniff	ssniff	ssniff	ssniff
Product no.	S9139-E028	S9139-E025	S9139-E032	V53x R/M-H auto	
ME [MJ/kg]	15,1	29,7	29,7	12,9	
Crude Protein [%]	16,1	8,1	8,1	19,0	
Crude ash [%]	4,5	4,4	4,4	6,4	
Crude fiber [%]	10,0	9,9	9,9	4,9	
Crude fat [%]	7,1	74,6	74,6	3,3	
LCT [% of crude fat]	6,8	74,6	49,3	3,25	
C8 [% of crude fat]	0	0,1	15,1	0	
C10 [% of crude fat]	0	0,3	10,2	0	
Sugar [%]	6,0	1,0	1,0	4,7	
Starch [%]	51,2	0	0	36,5	
Others [%]	5,1	2,0	2,0	25,2	

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