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The effects of ketogenic diet and fasting on inflammation in mice

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Abstract

Background: It has been proposed that elevated blood concentrations of the ketone body, β-hydroxybutyrate (BHB) provoked by ketogenic diets and/or fasting can dampen inflammation and have beneficial impact in reducing disease. Inflammation is the complex physiological response by the immune system. On one hand acute inflammation is crucial in creating a swift response towards harmful stimuli such as pathogens, mediators from damaged cells, or irritants and to repair damaged tissue caused by inflammation. Prolonged and chronic inflammation is on the other hand undesirable and causes a physiological state of constant degradation and repair in tissues. This dysregulation is associated with higher prevalence of chronic diseases such as cancer, diabetes, autoimmune- and neurodegenerative diseases. The transcriptional regulator NF-κB and NLRP3 inflammasome are central regulators of inflammation.
Aim: Our goal in this project was to investigate if ketogenic diets alone or combined with fasting, could reduce the severity of an LPS-induced acute inflammation in mice compared with a high-fat control group.

Methods: 30 transgenic NF-κB luciferase mice were separated into 3 groups. The groups were fed 1) a high-fat control diet (CD), 2) a ketogenic diet (KD) for three weeks or 3) ketogenic diet for three weeks with a final over-night fasting (KDF). The degree of inflammation was assessed by measuring NF-κB activity by *in vivo* and *ex vivo* imaging using luciferase as reporter gene. Additionally, cytokines in blood were measured. Consecutive measurements of blood glucose and ketones were carried out during the experiment. Also feed consumption, weight and body composition were assessed.

Results: Our results show that KD suppresses LPS-induced acute inflammation by lowering NF- κB activity in liver compared to CD, and secretion of TNF-α and IL-6 in comparison to KDF. The genders reacted differently to KD, whereas females maintained higher blood BHB levels compared to males and showed significantly less plasma IL-6 secretion and liver NF-κB activity compared to males. Males maintained higher non-fasting blood glucose levels by adherence to KD compared to females. Bodyfat, but not weight and lean mass increased in all groups.

Conclusion: We found that KD, but not KDF, reduced inflammatory markers in the animals. KD may provide some anti-inflammatory properties, but further research is needed to elucidate the effect of KD on inflammation.

Sammendrag

Bakgrunn: Det har blitt foreslått at forhøyede blodkonsentrasjoner av ketonlegemet, β-hydroksybutyrat provosert av en ketogen diett og/eller fasting kan dempe betennelse og ha gunstig effekt på å redusere sykdom. Betennelse er den komplekse fysiologiske responsen fra immunsystemet. På den ene siden er akutt betennelse avgjørende for å skape en rask respons mot skadelige stimuli som patogener, mediatorer fra skadede celler eller irritanter og for å reparere skadet vev forårsaket av betennelse. Langvarig og kronisk betennelse derimot er uønsket og forårsaker en fysiologisk tilstand med konstant nedbrytning og reparasjon i vev. Denne dysreguleringen er assosiert med høyere forekomst av kroniske sykdommer som kreft, diabetes, autoimmune- og nevrodegenerative sykdommer. Transkripsjonsregulatoren NF-κB og NLRP3 inflammasomet er sentrale regulatorer av inflammasjon.

Mål: Målet vårt var å undersøke om ketogene dietter alene eller kombinert med faste kunne redusere alvorlighetsgraden av en LPS-indusert akutt betennelse hos mus sammenlignet med en kontrolldiett med høyt fettinnhold.

Metoder: 30 transgene NF-κB luciferasemus ble separert i 3 grupper. Gruppene ble matet med 1) en fettrik kontrolldiett, 2) en ketogen diet i tre uker eller 3) ketogen diet i tre uker med en siste faste over natten. Graden av inflammasjon ble vurdert ved å måle NF-κB-aktivitet ved *in vivo* og *ex vivo* avbildning ved bruk av luciferase som reportergen. I tillegg ble cytokiner i blodet målt. Påfølgende målinger av blodsukker og ketoner ble utført under forsøket. Fôrforbruk, vekt og kroppssammensetning ble også vurdert.

Resultater: Våre resultater viser at KD undertrykker LPS-indusert akutt betennelse ved å senke NF-κB-aktivitet i lever sammenlignet med CD, samt sekresjon av TNF-α og IL-6 sammenlignet med KDF. Kjønnene reagerte ulikt på KD, hvorav hunner opprettholdt høyere BHB-nivåer i blodet sammenlignet med hanner og viste betydelig mindre plasma IL-6-sekresjon og NF-κB-aktivitet i lever sammenlignet med hanner. Hanner opprettholdt høyere ikke-fastende blodsukkernivåer fra KD sammenlignet med kvinner. Kroppsfett økte i alle gruppene, men verken vekt eller muskelmasse endret seg.

Konklusjon: Vi observerte at KD, men ikke KDF reduserte inflammatoriske markører i dyrene. KD kan muligens gi noen anti-inflammatoriske egenskaper, men ytterligere forskning er nødvendig for å belyse effekten av KD på inflammasjon.

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Abbreviations

AcAc	Acetoacetate
ACT-CoA	Acetoacetyl-CoA
BHB	β-hydroxybutyrate
BC	Body composition
BDH	β-hydroxybutyrate dehydrogenase
CD	Control diet
CPT-1	Carnitine palmitoyltransferase 1
DA	Detection antibody
DAMPs	Damage-associated molecular patterns
FGF21	Fibroblast growth factor 21
FPN	Freestyle Precision Neo
GPCR	G-protein coupled receptors
HDAC	Histone deacetylase
HDLc	High-density lipoprotein cholesterol
HFD	High-fat diet
HMG-CoA	Hydroxymethylglutaryl-CoA
HMGCS2	Hydroxymethylglutaryl CoA synthase
IFN-γ	Interferon gamma
IKK	Inhibitory kappa B kinase
IL	Interleukin
IκB	Inhibitory kappa B complex
Kcals	Kilocalories
KD	Ketogenic diet
LDLc	Low-density lipoprotein cholesterol
LPS	Lipopolysaccharide
mTORC1	Mammalian target of rapamycin complex 1
MCT	Monocarboxylic acid transporter
MCTG	Medium chain triglyceride
NBS	Nucleotide-binding site
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa B

NIK	Nuclear factor kappa B inducing kinase
NLRP3i	NACHT, LRR, and PYD domains-containing protein 3 inflammasome
NOX	NADPH oxidase
PAMPs	Pathogen-associated molecular pattern
PG	Picturing group
PPAR	Peroxisome proliferator activated receptor
PRR	Pattern-recognition receptors
PUFA	Polyunsaturated fatty acids
RCTs	Randomized controlled trials
RFP	Remaining feed particle
ROI	Region of interest
ROS	Reactive oxygen species
SAPE	Streptavidin-phycoerythrin
SCOT	Succinyl-CoA-3-oxaloacid CoA transferase
SFA	Saturated fatty acids
SOD	Superoxide dismutase
TC	Total cholesterol
TCA	Tricarboxylic acid cycle
TCAL	Total daily caloric value
TD-NMR	Time-domain nuclear magnetic resonance
TNF-α	Tumor necrosis factor α

1. INTRODUCTION

Inflammation is the immunological response towards pathogens and tissue injury (Singh et al., 2019). With inflammation being an important driver for a variety of modern diseases such as cancer, neurodegeneration and autoimmune diseases, more research is invested into the nutritional aspects for potential therapies. As early as the 1920's, a low-carbohydrate, high-fat diet known as a ketogenic diet (KD) was used by physicians to treat epilepsy. KD, as well as intense exercise and prolonged fasting induces physiological ketosis, where the production and metabolism of ketone bodies increases. The last decade, researchers all over the world have gained an increased interest in ketosis focusing on understanding the physiological consequences of elevated levels of ketone bodies.

Notably, within in the world of dieters and health-cautious people, a popular and new trend, yet ancestral and cultural-traditional, is the implementation of periodic eating patterns and intermittent fasting. Intermittent fasting, in similarity with low-carbohydrate and ketogenic diets, induces physiological ketosis in humans (Veech, 2004). Ketogenic diets was implemented in the 1920's as a strategy to treat epileptic seizures (Wheless, 2008). Even though the diet is not as common in treating epilepsy these days due to the discovery of anti-epileptic drugs, there has been an increase in scientific interest of different aspects of the diet. In ketosis, ketone bodies dominate as an energy providing source. The ketone bodies consist of β-hydroxybutyrate (BHB), acetoacetate (AcAc) and acetone, where BHB is the most abundant one. In addition to functioning as an energy providing molecule, BHB also possess some unique signaling properties, some which act anti-inflammatory.

1.1 Fasting – History, Culture, and Health

The term fasting is generally used to describe the voluntary abstinence from food and drinks, typically lasting for longer than an overnight fast (Visioli et al., 2022). Ever since the early times, fasting has been advocated as a strategy for spiritual development and health management. The great philosophers in the ancient Greece extolled the practice of fasting (Kerndt et al., 1982). In certain religions, such as Islam, fasting is implemented from dusk to dawn during the month of Ramadan (Alghafli et al., 2019), while Greek Orthodox Christians fast prior to Christmas and Easter (Trepanowski & Bloomer, 2010). In the last 10-15 years, different methods of fasting have popularized as a strategy to improve several parameters of

health (Hoddy et al., 2020). Alternate-day fasting, time-restricted feeding or prolonged fasting are some examples of fasting strategies (Crupi et al., 2020; Hoddy et al., 2020). Improvements in insulin levels and glucose levels (Carlson et al., 2007; Heilbronn et al., 2005; Horne et al., 2013) reduced fat mass (Catenacci et al., 2016; Stekovic et al., 2019) and decreases in fasting triglycerides (Chow et al., 2020; Hutchison et al., 2019; Sundfør et al., 2018) have been shown in human studies. However, complications with gallstone formation, hyperuricemia, and adrenal insufficiency are possible scenarios during prolonged fasts, making the diet trend a seemingly two-edged sword that needs to be practiced with caution (Chihaoui et al., 2017; Drenick et al., 1964; Runcie & Thomson, 1969; Venneman & van Erpecum, 2006).

1.1.1 Physiological Effects of Fasting

In the fed state, glucose is converted to glycogen and stored in hepatic cells. The glycogen synthesis is dependent on the enzyme glycogen synthase, which is stimulated by insulin. In the fasted state, insulin secretion is downregulated while glucagon secretion is increased, leading to inhibition of glycogen synthase while activating glycogen phosphorylase (Rui, 2014). During fasting, the liver releases glucose through the process of glycogenolysis. As fasting duration increases, hepatic cells synthesize glucose by gluconeogenesis from substrates such as lactate, pyruvate, glycerol and glucogenic amino acids. Moreover, in the fasted state when glucose levels are low, hepatic β-oxidation of fatty acids is increased and generation of ketone bodies are initiated (Rui, 2014).

1.2 The Rise of Ketogenic diets

The KD, a high-fat, low-carbohydrate, and moderate protein diet were originally developed as a fasting-mimicking diet to treat epilepsy in children in the 1920's (Wheless, 2008). The conventional KD consists generally of about 2% carbohydrate, 90% fat and 8% protein of the total caloric value (TCAL), even though there are different macronutrient modifications to the diet, allowing for more flexibility and palatability (Allen et al., 2014). Some examples of different types of KD are The Classical KD, medium-chain triglyceride (MCTG) KD and Modified Atkins KD (Wells et al., 2020). Since the last decade, an explosion of interest in both utilization and science behind the diet has risen. The KD has been accepted as an alternative treatment to epilepsy, in patents who does not respond adequately to medical treatment (Ułamek-Kozioł et al., 2019). Additionally, the diet has been demonstrated to reduce insulin and glucose levels in many studies (Ahmed et al., 2020; Michalczyk et al., 2020; Mohorko et al., 2019). In clinical trials for obese patients, the KD has been shown to

contribute to weight loss and BMI decrease (Dashti et al., 2007; Hallberg et al., 2018; Samaha et al., 2003). Pieces of evidence suggests that the diet may be beneficial for patients with neurodegenerative disorders such as Alzheimer's disease and Parkinson's disease, possibly due to the altered metabolic effect (Broom et al., 2019; Reger et al., 2004; Van der Auwera et al., 2005; Vanitallie et al., 2005).

A recent study published in Cell investigated the effect of KD on gut microbiome and found some interesting results (Ang et al., 2020). The study involved both mice and human adults following a KD, high-fat diet (HFD) or low-fat diet for 8 weeks. Ang and his colleagues found that KD alters the gut microbiota in a manner that facilitates the reduction of TH17 cells, primarily through the reduction of *Bifidobacterium*. This observation was specific to KD, and not HFD. Since the effect was KD-specific, the researchers hypothesized that the effect was due to one of the ketone bodies produced during adherence to a KD. To test this, they further isolated a strain of *Bifidobacterium* from human stool samples and applied the ketone body BHB directly to the isolated samples and observed a dose-dependent reduction in growth of *Bifidobacterium,* thus strengthening their hypothesis. Ang and colleagues concluded that the impact of the KD on microbiota may be beneficial or detrimental depending on the context.

Recent advances in oncotherapy have also suggested KD to be helpful in metabolic cancer therapy primarily due to reduced insulin signaling and reduced glucose availability (Weber et al., 2018). In tumor cells, the metabolism is altered to dramatically facilitate uptake of glucose to produce lactate, even in the presence of oxygen. This phenomenon is known as the Warburg effect (Liberti & Locasale, 2016; Warburg et al., 1927). As some cancer cells lack ketolytic enzymes, these cancer cells may have a reduced ability to metabolize the ketone substrates that is endogenously produced from a KD (Zhang et al., 2018). Several mice studies show reduced tumor growth and increased survival time for mice following the diet in combination with a standard cancer treatment regimen (Allen et al., 2013; Hopkins et al., 2018; Otto et al., 2008; Poff et al., 2013).

However, the typical KD contains a significant amount of saturated fatty acids (SFA). According to the American Dietary Guidelines for 2020 - 2025, saturated fats should be limited in order to reduce the risk of future heart disease (Phillips, 2021). High intake of SFA is associated with an increase in low-density lipoprotein cholesterol (LDLc) (Mensink & Organization, 2016). Long term exposure of high levels of LDLc, typically > 190 mg/dL is a well-known risk factor for cardiovascular disease (Vallejo-Vaz et al., 2017). A meta-analysis

of randomized controlled trials (RCTs) by Bueno and colleagues on obese patients following specifically a KD for at least 12 months, found a significant increase in LDLc and high-density lipoprotein cholesterol (HDLc) (Bueno et al., 2013). However, a more recent meta-analysis of RCTs did not show any significant difference of LDLc and HDLc between KD and a balanced diet (López-Espinoza et al., 2021). The patients in this study were also obese, but the trial duration was dramatically shorter, with a duration criterion of minimum 2 weeks. As in a meta-analysis on athletes following KD for an average of 5 weeks, certain ergogenic parameters and blood samples were assessed. Total cholesterol (TC) in KD athletes were increased in comparison to control groups, however, any conclusions on LDLc levels were not reported due to limited data (Lee & Lee, 2021), but Urbain et al. found increased LDLc and TC in healthy adults that followed a KD over 6 weeks (Urbain et al., 2017). Another study done on 20 healthy adults following either a high-polyunsaturated fat (PUFA) KD, or a high-SFA-KD for 5 days found that the SFA-KD group had significantly higher blood LDLc levels compared to the PUFA-KD (Fuehrlein et al., 2004). Some SFA, such as palmitic acid (C16:0) and lauric acid (C12:0) have been demonstrated to activate proinflammatory pathways in cell culture studies (Huang et al., 2012; Lee et al., 2003; Wong et al., 2009). A more recent study demonstrated that mice fed a diet high in C16:0 developed cartilage lesions in knee joints and an increased expression of cellular stress markers (Tan et al., 2021). Other adverse effects of the KD, such as gastrointestinal issues, hyperlipidemia, hyperuricemia, and lethargy have been reported (Cai et al., 2017). Due to the adverse effects and possibly elevations on LDLc levels, the KD may not benefit healthy adults, but may provide some benefits for people with certain medical conditions.

1.3 Ketosis, ketogenesis, induction and metabolism

As mammals have evolved through periods of starvation and food scarcity, the ability to adapt to the environment has been crucial for survival. In response to glucose deprivation, the requirement for an alternative fuel source is needed. This is where ketone bodies provide their usefulness. The ketone body acetone, acetoacetate and BHB are small, water-soluble, lipid-derived molecules produced primarily in the liver from fatty acids (Dhillon & Gupta, 2022). It is important to distinguish between nutritional ketosis and the pathophysiological state of ketoacidosis seen in type 1 diabetes, where ketone bodies are severely elevated (10-25 mmol/L) and the process of ketogenesis results in acidification of the blood (Green & Bishop, 2019). Even though small amounts of ketone bodies are constantly produced within the body,

the state of nutritional ketosis is typically defined by an elevation of circulating BHB levels (Volek & Phinney, 2012).

Ketogenesis occurs primarily in hepatocytes where fatty acids are transported into the mitochondria by carnitine palmitoyltransferase (CPT-1) and metabolized into acetyl-CoA via β-oxidation. The enzyme thiolase condenses acetyl-CoA into acetoacetyl-CoA (ACT-CoA), which is further converted to hydroxymethylglutaryl-CoA (HMG-CoA) via HMG-CoA synthetase 2, which is the rate-limiting enzyme in production of ketone bodies (Hegardt, 1999). HMG-CoA lyase (HMGCL) converts HMG-CoA to acetoacetate, which can be converted to acetone via a non-enzymatic decarboxylation or to BHB via β-hydroxybutyrate dehydrogenase (BDH) (Dhillon & Gupta, 2022). Transcriptional regulation of HMGCS2 occurs in two different pathways. The forkhead box transcription factor FOXA2 regulates the transcription of HMGCS2, and its activity is dependent on the antagonistic hormones insulin and glucagon. Insulin signaling leads to FOXA2 inactivation, while glucagon leads to FOXA2 activation by acetylation via the cofactor p300 (von Meyenn et al., 2013). The second regulator of HMGCS2 is fibroblast growth factor 21 (FGF21), which is dependent on peroxisome proliferator activated receptor α (PPARα). PPARα is suppressed under mammalian target of rapamycin complex 1 (mTORC1), and thus inhibition of mTORC1 is required for the induction of PPARα to induce FGF21 (Badman et al., 2007; Badman et al., 2009). HMGCS2 can also be regulated by post-translational modifications of succinylation or acetylation (Quant et al., 1990; Shimazu et al., 2010).

In glucose deprived circumstances, insulin is reduced while glucagon secretion is increased (Rui, 2014). Glucagon and other metabolic hormones regulate β-oxidation of fatty acids, leading to an increase in the fatty acid derived metabolite acetyl-CoA. As the acetyl-CoA pool increases, the citric acid cycle (TCA) becomes oversaturated with acetyl-CoA. This causes a "spillover" of acetyl-CoA, as it eventually exceeds either the capacity of citrate synthase activity, or the oxaloacetate availability to form citrate. The excess acetyl-CoA is thus used for biosynthesis of ketone bodies (Puchalska & Crawford, 2017). An illustration of the main steps involved in ketogenesis is shown in Figure 1.1 below.

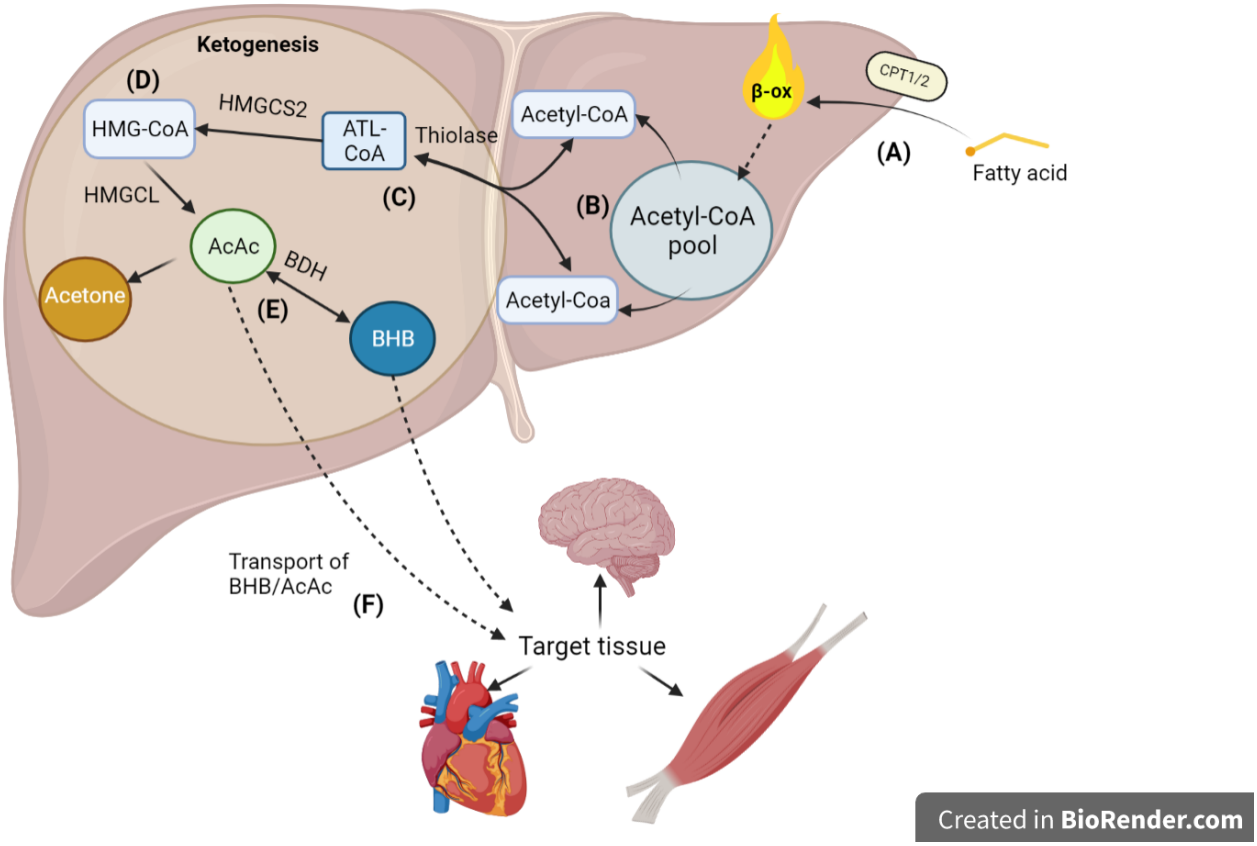

Figure 1.1: Hepatic ketogenesis. (A): The transport of fatty acids into hepatocytes are dependent on carnitine palmitoyltransferase 1 or 2 (CPT1/2). Fatty acids undergo β-oxidation which produces acetyl-CoA to fuel the pool of acetyl-CoA **(B). (C):** When the pool of acetyl-CoA exceeds the capacity of the citric acid cycle (TCA) to form citrate, 2 acetyl CoA molecules are condensed by thiolase to create acetoacetyl-CoA (ACT-CoA). **(D)**: ATL-CoA is converted to hydroxymethylglutaryl-CoA (HMG-CoA) by HMG-CoA synthetase 2 (HMGCS2) and is further converted to acetoacetate (AcAc) by HMG-CoA lyase (HMGCL). **(E)** AcAc may spontaneously form acetone or is reduced to β-hydroxybutyrate (BHB) by BHB dehydrogenase (BDH). **(F)**: BHB and AcAc is transported to its extrahepatic target tissues such as brain, heart, or skeletal muscle where it eventually enters TCA to produce energy. The figure is created in BioRender.com, with inspiration from Puchalska and Crawford (Puchalska & Crawford, 2017).

After hepatic ketogenesis, the ketone bodies are transported by blood circulation to their target tissues (brain, skeletal muscle and heart). The transport mechanisms for BHB are not fully understood. However, the monocarboxylic acid transporters (MCT) MCT1 and MCT2 have been found to carry BHB across the blood-brain barrier (Pellerin et al., 2005). Upon arriving to their extrahepatic tissues, BHB is converted back into acetoacetate via BDH, and acetoacetate is converted back to acetyl-CoA by the enzyme succinyl-CoA-3-oxaloacid CoA transferase (SCOT) before it can enter the TCA (Puchalska & Crawford, 2017). Extrahepatic tissues that favor ketone body metabolism during ketosis, are mainly brain, heart, and skeletal muscle (Balasse & Féry, 1989; Cotter et al., 2013; Kashiwaya et al., 1994). Ketone bodies

may constitute about 60% of the brains energy requirements during ketosis (Owen et al., 1967).

During the transition to ketosis, a physiological phenomenon of adapting to a metabolic switch occurs. The metabolic switch, in this sense, is the reduced utilization of glucose from glycogenolysis, to an increased fatty acid β-oxidation and ketone body oxidation (Anton et al., 2018). In liver cells, this metabolic switch is regulated partly by sirtuins (Liu et al., 2008). SIRT1 increases the oxidation of fatty acids by deacetylating peroxisome proliferator-activated receptor gamma coactivator 1-alpha and activating PPARα (Purushotham et al., 2009; Rodgers et al., 2005). PPARα promotes oxidation of fatty acids in mitochondria and peroxisomes, while also being an important regulator for bile acid biosynthesis, transport, and secretion (Kersten, 2014; Li et al., 2012). SIRT1 has also been shown to be activated in muscle cells during fasting and exercise through AMP-activated protein kinase signaling, which senses the presence of a high AMP to ATP ratio to restore cellular ATP (Cantó et al., 2010; Hardie, 2007). Additionally, the expression of SIRT3 in muscle cells is induced by fasting and may protect muscle cells against oxidative stress by activating SOD2 (Katwal et al., 2018; Palacios et al., 2009). The expression of the mediators in ketogenesis, HMGCS2 and BDH, are increased through upregulation of PPARγ as seen in mice fed high-fat or KD (Sikder et al., 2018; Zhong et al., 2021). An increased number of mitochondria in rat hippocampus has also been observed by feeding the animals a KD for at least 4 weeks (Bough et al., 2006). The upregulation of ketogenic enzymes and mitochondrial biogenesis suggests an increased efficiency towards ketone and fatty acid metabolism in prolonged ketosis. A study done by Appleton and DeVivo illustrated that rats required a total of 20 days to reach maximum benefit from the anti-convulsant effects of a high-fat diet, suggesting that several weeks may be required to fully adapt to the diet for rodents (Appleton & DeVivo, 1974).

With a brief understanding of ketogenesis and the ketogenic metabolism, we will dive into the most abundant ketone body, in which seemingly exerts some intriguing properties with respect to inflammation.

1.4 Beta-hydroxybutyrate, properties and signaling

Apart from yielding ATP after conversion to acetyl-CoA, several signaling mechanisms for BHB has been proposed. These include binding to cell-surface receptors, inhibition of enzymes, and modulation of ion channel activity. This is elucidated below.

1.4.1 GPR109a agonism

Amongst the three classes of cell-surface receptors, G-protein coupled receptors (GPCRs) mediates most cellular responses to extracellular stimuli (Alberts, 2015; Weis & Kobilka, 2018). Upon binding of a ligand to a GPCR, the receptor changes its conformation, and a trimeric GTP-binding protein (G protein) is activated. This alters the conformation of the G protein and releases GDP from its nucleotide-binding site (NBS). A further binding of GTP to the NBS promotes closure of the NBS, which triggers a conformational alteration that cause dissociation of the α-subunit from the GPCR and the βγ-subunit. This sends a signal, which can regulate the activity of downstream molecules. The GPCR stays active if a ligand is bound to it, allowing catalyzation of many G-protein molecules (Alberts, 2015).

One of the GPCRs prevalent in enterocytes, adipocytes and immune cells are GPR109a (Blad et al., 2012; Ganapathy et al., 2013). GPR109a activation has been demonstrated to have a suppressive effect on several aspects of inflammation, including LPS-induced NF-κB activity in mice (Rahman et al., 2014; Sivaprakasam et al., 2016; Xu et al., 2017), but also a promotive effect on NLRP3 inflammasome activation in the colon (Macia et al., 2015). Activation of GPR109a by butyrate or niacin has been demonstrated to suppress colonic inflammation and carcinogenesis in mice, possibly through the promotion of IL-10- T-cells (Singh et al., 2014). Singh et al. found that GPR109a was essential in butyrate-mediated induction of IL-18 in colon. Moreover, mice deficient in GPR109a had an increased risk for developing colon cancer (Singh et al., 2014). Butyrate and BHB has a quite similar chemical structure, and notably BHB has been identified as a GPR109 ligand (Soudijn et al., 2007; Taggart et al., 2005). Taggart and colleagues found that physiological achievable serum concentrations of BHB was able to inhibit lipolysis from adipocytes via GPR109a, likely as a strategy to conserve energy expenditure during starvation (Taggart et al., 2005).

GPR109a activation by niacin recruits β-arrestin to the cell membrane (Walters et al., 2009), and Witherow et al. found that β-arrestin in partner-binding with IkBα stabilizes IkBα and prevents the nuclear translocation of NF-κB (Witherow et al., 2004). This finding suggests a possible crosstalk mechanism between GPR109a activation and NF-κB activity.

1.4.2 Cytosolic K^+ modulation

A common trigger for the activation signal to NLRP3 assembly, is the permeation of the cell membrane to K^+ (Muñoz-Planillo et al., 2013). A study done by Youm et al. investigated the effects of BHB on NLRP3 inflammasome activation through different mechanisms, and found that BHB prevented intracellular K^+ decline after treating cell cultures with ATP, MSU or ceramides, which all are known agonists for K^+ efflux (Kelley et al., 2019; Youm et al., 2015). The researchers concluded that BHB blocks NLRP3 inflammasome activation through regulating an unknown upstream agonist for K^+ efflux in macrophages. The precise mechanism of BHB in modulating K^+ efflux is unknown.

1.4.3 Epigenetic regulation by HDAC inhibition

In addition to being an agonist for mediators involved inflammatory responses, BHB has been proven to inhibit histone deacetylases (HDACs) and is therefore classified as an HDAC inhibitor (Li et al., 2021; Shimazu et al., 2013). HDACs are enzymes that control epigenetic regulations, through modifications on histones and other protein regulatory factors by removing acetyl groups on the proteins. In general, the modifications are associated with a repressive effect on gene expression (Ceccacci & Minucci, 2016). By inhibiting these enzymes, gene expression may increase in a favorable manner in respect to inflammation (Adcock, 2007). As of a study done by Shimazu et al. on mice, BHB inhibited HDAC 1, HDAC 2 and HDAC 3 at physiological achievable concentrations (<5.3 mmol/l) where inhibition of HDAC 1 and HDAC 2 appeared to result in acetylation of histones at the promoter of genes involved in resistance to oxidative stress, such as *FOXO3a* and *MT2*. Markers for oxidative stress such as lipid peroxidation were also measured, where a significant decrease ($P < 0.05$) of lipid peroxide in response to paraquat, a ROS accumulating inducer, was observed in BHB treated mice. Additionally, another marker for oxidative stress, 4-Hydroxynonenal, was completely suppressed in the BHB treated mice to the same ROS inducer, paraquat (Shimazu et al., 2013). One of the non-histone proteins that may be acetylated and affected by HDACs is the p65 subunit of NF-κB dimers. Acetylation of p65 reduces its ability to bind to the kB-DNA and HDAC3 has been identified as the HDAC responsible for p65 deacetylation (Kiernan et al., 2003; Leus et al., 2016). Moreover, BHB at physiologically concentrations of 5 mmol/L has been shown to inhibit HDAC3 in neurons, consistent with an increase in acetylated H3 histones (Sleiman et al., 2016). In human cardiac endothelial cells, BHB blood concentrations of 2 mmol/L was found to attenuate cardiac

endothelial decay by inhibiting HDAC3 and cause acetylation on the promoter for *CLDN5*, which codes for claudin-5, a tight-junction protein vital for maintaining integrity to endothelial cells (Greene et al., 2019; Li et al., 2021). However, there is no current evidence that BHB directly inhibits the p65 subunit through HDAC3 inhibition.

What makes the old lifestyle- and dietary practices relevant for today's challenges in health and disease? To get a clear idea of this relevance, we must first look into one of the commonalities seen in modern diseases.

1.5 Inflammation

With inflammation being an important driver for a variety of modern diseases such as cancer, type 2 diabetes, autoimmune- and neurodegenerative diseases, more research is being invested into the nutritional aspects for potential therapies (Glass et al., 2010; Mantovani et al., 2008; Matsushita et al., 2020; Solfrizzi et al., 2003). As inflammation is a necessary, yet complex and not fully understood immunological response in body tissues to cell damage and pathogens (Ferrero-Miliani et al., 2007), understanding the regulatory mechanisms of the immune system through immunological signaling pathways are crucial elements to tackle chronic or acute inflammation with a medicinal or preventative approach. Common regulatory mechanisms and mediators within the immune system involves the activation and suppression of transcriptional factors such as NF-κB, production- and secretion of cytokines and activation of inflammasomes (Ferrero-Miliani et al., 2007; Kelley et al., 2019). The activity and expression of these inflammatory mediators can be influenced by exogenous or endogenous compounds, while dietary interventions have been shown to affect inflammatory biomarkers in favorable ways (Chacko et al., 2010; George et al., 2010; Jahns et al., 2018; Ma et al., 2008; North et al., 2009).

Inflammation is the immunological response towards tissue injuries and infections (Majno & Joris, 2004). Acute inflammation is a short-term response with the purpose of healing, where leukocytes enter the site of injury to remove the inflammatory stimuli and repair the tissue. Chronic inflammation, on the other hand, is a dysregulated and prolonged response where tissue resides in a state of constant degradation and repair (Weiss, 2008). The latter is a rather obscure process and much less is known about the molecular and cellular pathways thereby. What is known, however, is that chronic inflammation is associated with many degenerative conditions and chronic diseases like cancer, atherosclerosis, allergies and autoimmune

diseases (Weiss, 2008). Central to different aspects of immune functions is the NF-κB pathway (Sun, 2017).

1.5.1 NF-κB pathway

NF-κB is a family of dimeric transcription factors central to stress and immune responses. The dimers are combined with two of the subunits p50, p52, p65/RelA, RelB and cRel (Mulero et al., 2019). In the inactivated state, NF-κB dimers retains latent in the cytosol associated with an inhibitory kappa B (IkB), or with the precursor proteins p100 and p105 (Sun, 2017). NF-κB is liberated from IkB by phosphorylation of inhibitory kappa B kinase (IKK) when triggered by an upstream agonist. The NF-κB pathway is divided into two, the canonical pathway and the alternative pathway. What primarily distinguishes the two pathways are the requirements for different IKK subunits (Lawrence, 2009; Mulero et al., 2019).

The canonical pathway is dependent on the NF-κB essential modulator (NEMO). NEMO is a scaffold protein that makes up a kinase complex together with the two kinases IKKα and IKKβ. Upon activation, the IKK complex phosphorylates IkB and marks it for ubiquitylation and degradation in proteasomes. When IkB is degraded, NF-κB is liberated and free to translocate into the nucleus where it binds to DNA for target gene expression (Hoffmann et al., 2002; Mitchell et al., 2016). The pathway is activated by a series of signals related to inflammatory stimulus, such as pathogen-associated molecular patterns (PAMPs) and inflammatory cytokines (Mitchell et al., 2016). The canonical pathway will be emphasized in this paper and is illustrated in Figure 1.2.

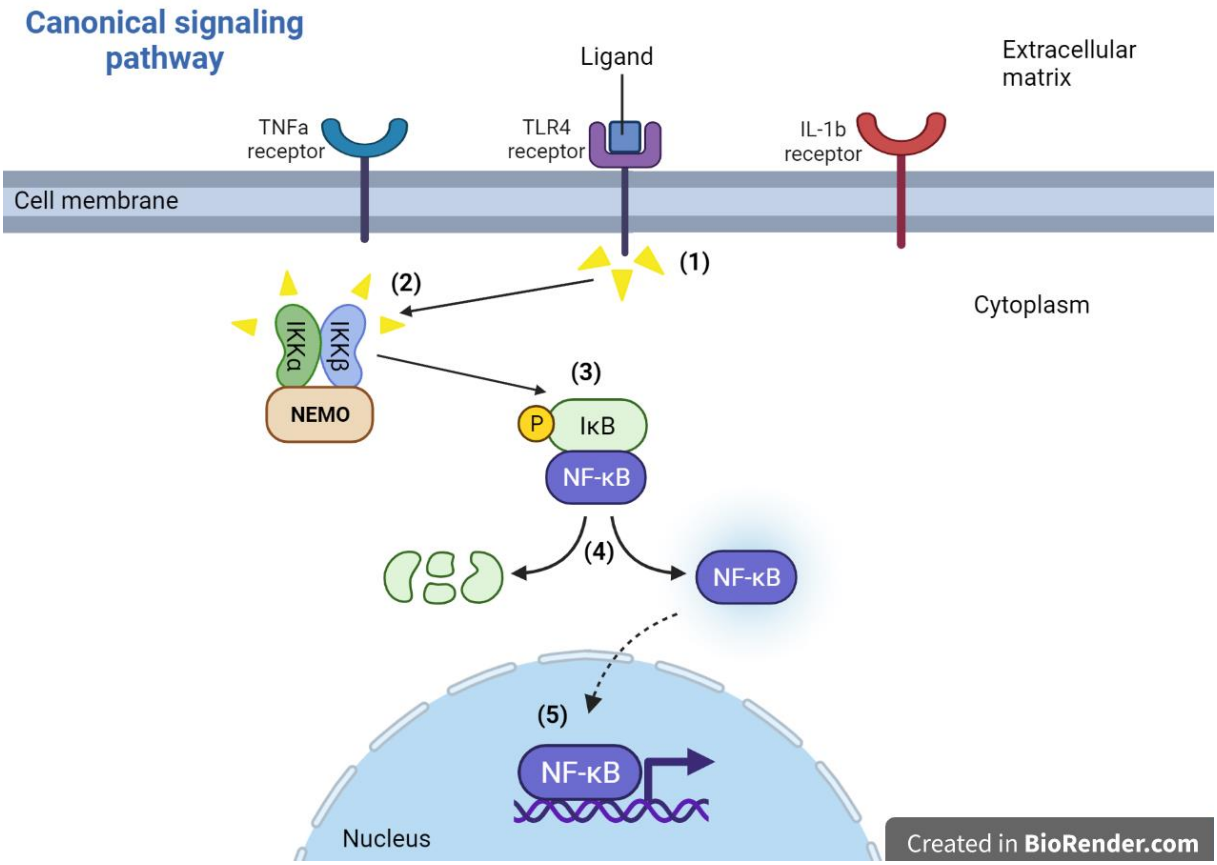

Figure 1.2: NF-κB canonical signaling pathway (simplified). Upon ligand-receptor interaction on the cell surface, a signal is transduced from the stimulated receptor **(1)** to activate the IKK-complex **(2)**. The kinase activity of the IKKβ subunit targets IkB for phosphorylation **(3)**. The phosphorylated IkB undergoes ubiquitylation and is degraded which liberates the NF-κB dimer **(4)**. The liberated NF-κB dimer rapidly translocates into the nucleus and binds to κB-binding sites **(5)**, where it increases transcription of target genes. The figure is created in Biorender.com, with inspiration from Mulero and colleagues (Mulero et al., 2019).

The alternative pathway is characterized by its independency of NEMO and IKKβ but is dependent on IKKα and NF-κB inducing kinase (NIK). This pathway induces the phosphorylation of p100 by IKKα, resulting in the activation of RelB/p52 heterodimers or p52 homodimers (Mulero et al., 2019). The activation of IKKα occurs by NIK, which is a central kinase to this pathway. NIK is later marked for degradation by the protein coding gene *TRAF3* (Liao et al., 2004). The alternative pathway has been shown to be involved in lymphoid organ development, B cell survival and differentiation of osteoclasts, but the pathway is yet to be fully understood (Dejardin, 2006; Novack, 2011; Sun, 2012).

It is important to mention that NF-κB can function both as an activator and a repressor for gene transcription. This fate is dependent on the subunits of the dimer and its combinations.

For instance, p50 and p65/RelA subunits have showed to play an important role in the repression of NF-κB target genes by studying the immune response to LPS-injections in homozygous and heterozygous mutant mice (Gadjeva et al., 2004). However, a dimer consisting of p50 and p65 subunits does promote gene transcription (Hayden & Ghosh, 2004). The explanation lies in the trans-activation domain, which only the three subunits RelA, RelB and cRel contains (Pereira & Oakley, 2008). The subunits and its combinational options make the NF-κB signaling pathway a complex, tightly regulated system.

A convincing number of studies imply that dysregulations within the NF-κB pathway are prevalent in human cancers, autoimmune diseases and neuroinflammation (Bassères & Baldwin, 2006; Cilloni et al., 2007; Ilchovska & Barrow, 2021; Jost & Ruland, 2007; Shabab et al., 2017). A study on a mouse model of vascular dementia done by Saggu and colleagues investigated the role of NF-κB pathway in astrocytes and found that transgenic inhibition of the pathway offered protection from neurodegeneration. By exposing the mice to chronic hypoperfusion and using transgenic inhibition of NF-κB, they could compare the NF-κB activity to the functionality of elements to neurodegeneration such as axonal loss, gliosis, white matter deterioration and demyelination. Upon transgenic inhibition of NF-κB, a general protection against neurodegeneration was observed and so the researchers concluded that the study demonstrated how chronic activation of NF-κB was a central component of disease progression in their mouse model (Saggu et al., 2016).

With its central role in inflammation and prevalence in essentially all cell types (Hoffmann & Baltimore, 2006), the NF-κB pathway makes up an interesting target for potential therapies and prevention against chronic inflammation. The first step to understand the drivers of this comprehensive pathway, is to identify the cell surface receptors and its agonists.

1.5.2 NF-κB agonists

The canonical pathway can be activated through several signal molecules binding to different pattern-recognition receptors (PRRs). One of the PRRs, the toll-like receptor 4 (TLR4), recognizes pathogen-associated molecular patterns (PAMPs) such as LPS, which in this experiment will be implemented to induce an acute inflammatory response (Ciesielska et al., 2021). Upon ligand-receptor interaction, the IκK complex formation is initiated, allowing the downstream activation of NF-κB (Mitchell et al., 2016). Pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin 1-beta (IL-1β) are recognized by the TNF receptor and IL-1β receptor, respectively (Mitchell et al., 2016). These cytokines are produced

and secreted by several cell types, but the major contributor are macrophages and T-helper cells (Zhang & An, 2007). The production and synthesis of these pro-inflammatory cytokines originates from inflammatory multiprotein oligomer complexes, known as inflammasomes (Mariathasan et al., 2004). One of the inflammasomes, the NACHT, LRR, and PYD domains-containing protein 3 inflammasome (NLRP3i), is of particular interest in this experiment.

1.5.3 NLRP3 inflammasome

A major inflammatory pathway for macrophages to eliminate any microbial infection or repair damaged tissue, is through activation of the NLRP3i (Zhao & Zhao, 2020). The NLRP3i consists of NLRP3 proteins that belongs to the nucleotide-binding and oligomerization domain-like receptors. Additionally, this inflammasome contains adaptor protein apoptosis-associated speck-like protein (ASC) and procaspase-1 (Inoue & Shinohara, 2013; Ito et al., 2015). The assembly of the NLRP3i is dependent on interactions between the components, which is regulated tightly by signals that, according to the two-signal model for NLRP3i, control two novel steps of assembly of the inflammasome complex (Kelley et al., 2019; Shao et al., 2015). The first step, also known as priming, requires a priming signal which is induced by PRRs recognizing any PAMPs or damage-associated molecular patterns (DAMPs), resulting in the downstream activation of NF-κB which up-regulates transcription of the inflammasome components and pro-interleukins (Bauernfeind et al., 2009; Franchi et al., 2012; Franchi et al., 2014). The second step, the activation, involves the oligomerization and assembly of the inflammasome components to form a complex. This results in activation of caspase-1, which matures proIL-1β and proIL-18 allowing the active cytokines to be secreted. The activation step is dependent on a wide range of stimuli, including ionic flux, mitochondrial dysfunction, reactive oxygen species (ROS) and lysosomal damage (Kelley et al., 2019; Mariathasan et al., 2006; Martinon et al., 2006; Qiu et al., 2019).

Ionic flux, in particular K+ cytosolic efflux, has been shown to activate NLRP3i in human macrophages and monocytes (Mariathasan et al., 2006; Muñoz-Planillo et al., 2013; Perregaux & Gabel, 1994; Walev et al., 1995). Walev and colleagues demonstrated the role of K^+ depletion on IL-1β maturation in human monocytes, by implementing an alpha-toxin that permeabilized the cell membrane for monovalent ions, allowing K^+ to permeate. By suspending the monocytes to a medium of high K^+ concentration, the researchers observed a total blockage of proIL-1β processing, while the opposite was observed when replacing K^+ in the medium with either a high Na^+, sucrose or choline concentration. The monocytes were

also stimulated with LPS for 1-2 hours to trigger proIL-1β synthesis and further transferred to a K^+ rich medium, which resulted in total suppression of IL-1β maturation. The researchers hypothesized that local K^+ concentration influences the activity of interleukin-1β-converting enzyme and possibly intracellular proteases (Walev et al., 1995). Other ionic events that are implicated in NLRP3i is the efflux of Cl- and influx of Na^+ (Schorn et al., 2011; Verhoef et al., 2005). In the study done by Schorn et al., IL-1β secretion was observed after treating monocytes with an agent that increases Na^+ influx. However, with an increase in intracellular Na^+ concentration also follows an increase in water content and cell swelling, which further dilutes the K^+ concentration. The researchers thus suspected that the NLRP3i activation was not a direct effect of the Na^+ influx, but rather the consequence of a drop in K^+ below a threshold value that activates the NLRP3i (Schorn et al., 2011). A more recent study by Yamanashi and friends investigated IL-1β and TNF-α levels after treating rats with BHB. The researchers used a single prolonged stress model over two weeks, to induce an anxiety-like behavior, which elevated the inflammatory cytokines IL-1β and TNF-α in the rats. They further administrated BHB subcutaneously into the rats to see if they could attenuate the cytokine response. IL-1β levels were not significantly affected, but there was a significant decrease in TNF-α levels after BHB administration (Yamanashi et al., 2020).

Despite being somewhat controversial, another event that has gained attention to the NLRP3i activation is the production of reactive oxygen species (ROS) (Zhou et al., 2011). ROS are oxygen derived metabolites that play important roles in maintaining homeostasis through different physiological processes. However, a prolonged exposure to high ROS concentration may cause damage to cellular components (Brieger et al., 2012). An enzyme that is commonly involved in the production of ROS in phagocytic cells, is NADPH oxidase (NOX), which is included in host defense, cellular signaling, gene regulation, cell differentiation, but also pathological processes (Bedard & Krause, 2007). By using a NOX2 knockout mouse model for traumatic brain injury, Ma and colleagues demonstrated a reduction in NLRP3i activation (Ma et al., 2017). The effect seemed to be tissue-specific whatsoever, and a few other studies did not find any effect on NLRP3i activation when implementing NOX inhibitors (van Bruggen et al., 2010; Zhong et al., 2013). Other researchers have focused on mitochondrial ROS through respiration as the possible main source of endogenous ROS production (Zhou et al., 2011), but Muñoz-Planillo and his crew failed to detect NLRP3i activation by stimulating the mitochondria with toxic agents that induce ROS production and concluded that ROS do not play a role in NLRP3i activation (Muñoz-Planillo et al., 2013). The poor evidence

amongst these studies suggests that ROS does not have a direct, if any effect at all on NLRP3i activation.

Even though more research and clarification on the different events involved in NLRP3i activation are to be determined, K^+ efflux remains generally as the most critically important and fundamental driver. A simplified, theoretical interplay between BHB, NF-κB and NLRP3i inflammasome is illustrated in Figure 1.3 below.

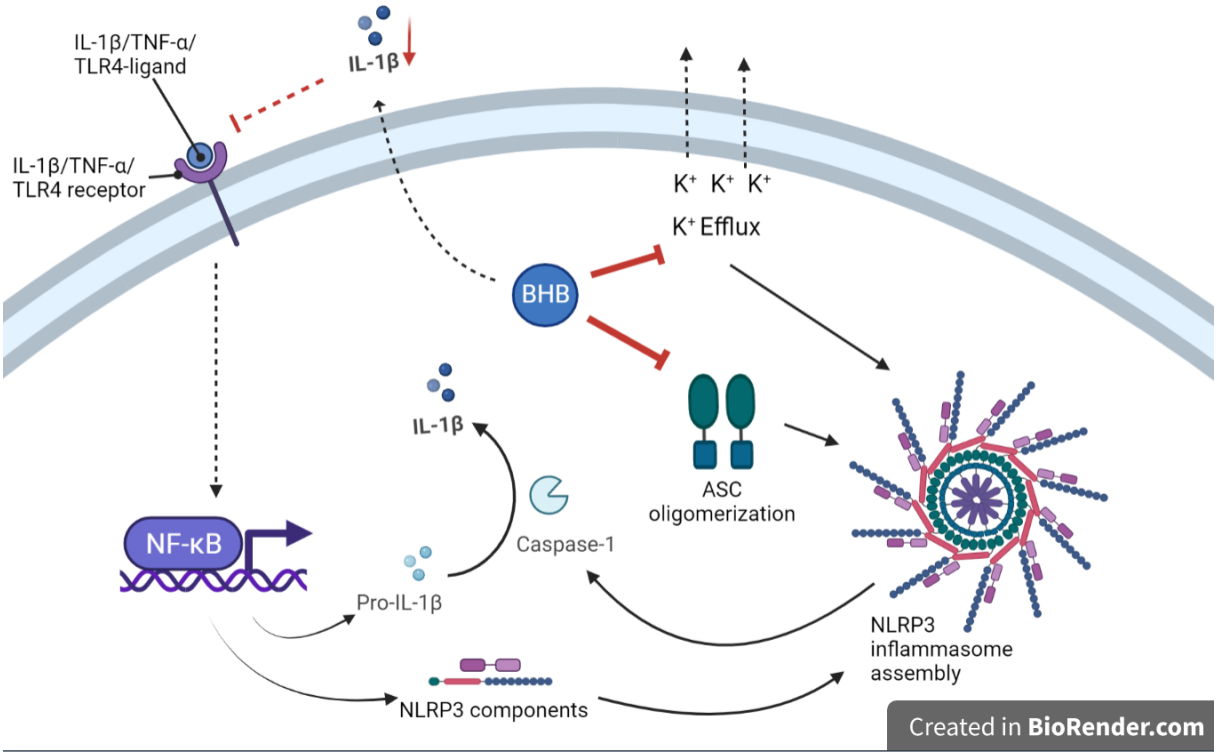

Figure 1.3: Theoretical interplay between BHB, NF-κB and NLRP3 inflammasome (simplified). *Under regular NACHT, LRR, and PYD domains-containing protein 3 inflammasome (NLRP3i) inducing circumstances (activation and priming signal required), a feedback mechanism between NLRP3i and Nuclear factor kappa B (NF-κB) ensures continuous assembly of the NLRP3i inflammasome through the transcription of NLRP3 components and pro-IL-1β. Pro-IL-1β is processed by Caspase-1 to IL-1β and secreted. IL-1β may bind to IL-1β receptors, initiating autocrine signaling. A common activation signal for NLRP3i inflammasome assembly is K^+ efflux. BHB suppresses adaptor protein apoptosis-associated speck-like protein (ASC) oligomerization and K^+ efflux through an unknown mechanism, further suppressing the assembly of NLRP3 inflammasome. Processing of pro-IL-1β is then suppressed and secretion of IL-1β is reduced, altering the autocrine feedback mechanism by reducing IL-1β dependent NF-κB signaling. The theory behind the figure is based of Youm and his colleagues' article (Youm et al., 2015). The figure is created in BioRender.com.*

1.6 Transgenic NF-κB reporter mouse

To investigate degree of inflammation in research models, many possibilities exist including assessing levels of proinflammatory cytokines in blood, mRNA levels of NF-κB target genes in specific organs or exploiting reporter genes whose activity reflect NF-κB activation. The latter approach has been used in this thesis. To this end transgenic mice have been developed that contain the gene encoding the firefly luciferase under the control of a classical NF-κB binding site/response element. These mice, named NF-κB-luc contain a transgenic gene construct with 3 NF-κB response elements connected to luciferase, present in all cells of the mouse. By introducing various proinflammatory stimuli into the mouse, it has been demonstrated that luciferase activity can be assessed by in vivo technology and in single organs, meaning that luciferase activity is translated into a light signal (bioluminescence) which can be monitored, and importantly enabling real time in vivo imaging of NF-κB activity. LPS administered to these mice will typically lead to a rapid increase in light production. By combining LPS with inflammatory modulators, this model can be used for testing efficacy of such modulators (Carlsen et al., 2002). Light emission (radiance) is typically measured as photon emission with a sensitive camera. Figure 1.4 illustrates the principle of using the mouse model to assess NF-κB activity.

The NF-κB transgenic C57BL/6J mice express the firefly gene luciferase under control of 3 NF-κB response elements. The luciferase-derived bioluminescence is thus strongly regulated in response to common inducers and modulators of NF-κB (Carlsen et al., 2002). By measuring radiance of photons from bioluminescence over a given period, NF-κB activity can be quantified with a bioluminescent capturing device.

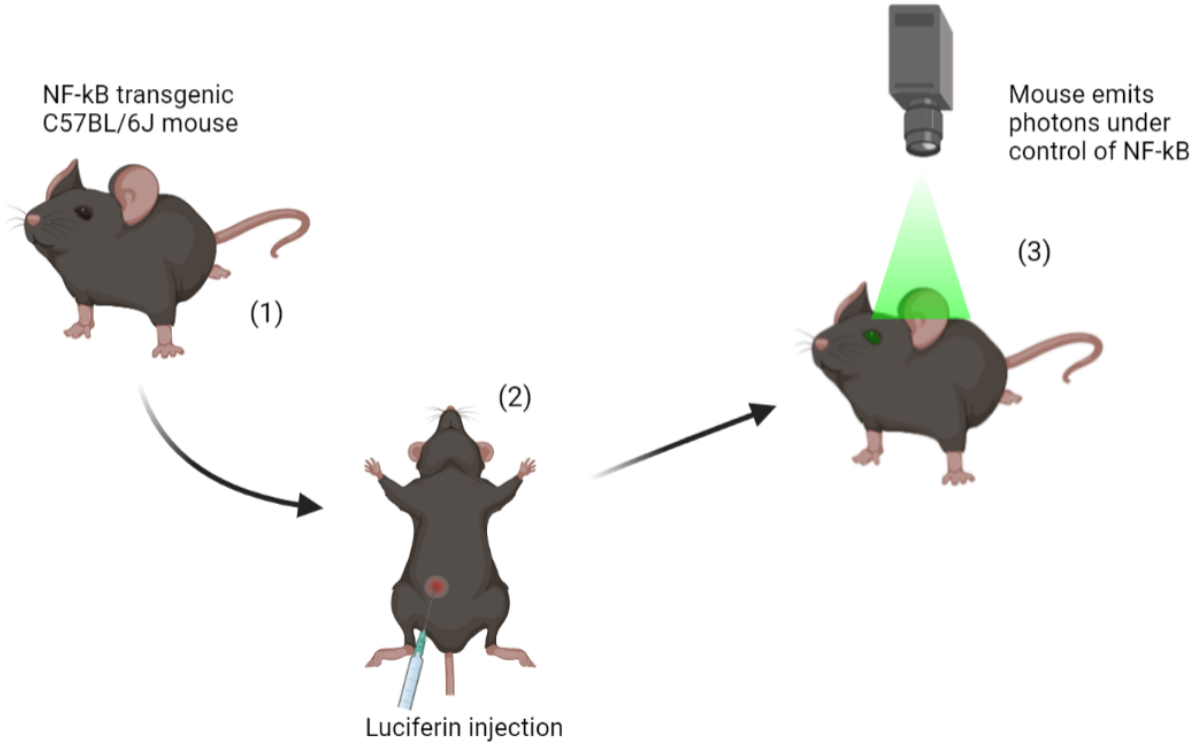

Figure 1.4: Principle of NF-κB mouse reporter function. In a NF-κB transgenic mouse, luciferase expression is dependent on NF-κB activity **(1)**. By injecting luciferin into the mouse **(2)**, luciferase initiates the reaction where luciferin is oxidized, and light is produced as one of the products. Light emission can be detected through a light-sensitive camera to assess NF-κB activity **(3)**. The figure is created in Biorender.com.

The luciferase-mediated reaction is illustrated in (1) below.

(1) $$\text{D-Luciferin} + O^2 + \text{ATP} \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{light}$$

1.7 Aim and rationales of the experiment

Adherence to KD or fasting causes physiological ketosis in humans and mice and thus increases circulating BHB, which has been shown to affect mediators in several inflammatory pathways. The NF-κB pathway and NLRP3 inflammasome assembly are of particular interest, in which we primarily hypothesize BHB to suppress the NF-κB signaling pathway and cytokine production indirectly by affecting the NLRP3i. To test this hypothesis, we fed mice with a ketogenic diet and a control diet that only differed in the amount of fat and presence of

carbohydrates. By using NF-κB-luc mice as reporter animals, we can monitor NF-κB activity with *in vivo* imaging through bioluminescence quantification. Cytokine profiling will be performed with a ProcartaPlex kit and its compatible Bioplex-200 systems.

The main aim of this experiment was to evaluate the anti-inflammatory effects of ketogenic diet and fasting possibly mediated by BHB. Specifically, we did the following to evaluate the physiological effects of the KD and fasting:

- Observed the dynamics of body composition parameters of the animals during the diet period
- Measured consecutive blood non-fasting glucose levels during the diet period
- Measured consecutive blood BHB levels during the diet period
- Investigated how NF-κB activity is affected by the diets, *in vivo* and *ex vivo* of liver and intestines
- Investigated how the cytokines IL-1β, IL-6, IL-10, IL-18, IFN-γ and TNF-α were affected by the diets

2 Materials

2.1 Solutions for NF-κB analysis

Luciferin	D-Luciferin, Firefly 15mg/mL PBS 1 M NaOH
LPS	

2.2 Solutions for blood sampling

0.5 M EDTA	50 mM final solution, pH 8

2.3 Solutions for anesthesia

Isoflurane	2,5%
ZRF cocktail	Zoletil Forte 250 mg Rompun 20 mg/ml Fentadon 50 µg/ml Sterile isotone NaCl

2.4 Diets and composition

Tabell 2:1: Ketogenic and control feed composition.

	KD		Control	
	gram%	**kcal%**	**gram%**	**kcal%**
Protein	18,1	10,0	15,4	10,2
Carbohydrates	0,8	0,6	23,4	14,9
Fat	63,4	89,4	45,6	74,7
Other (Vitamin mix)	4,3	0	4,2	0
Fibre	6,6		6,6	
Total	**93,09**	**100,00**	**95,2**	**99,9**
Ratio kcal/gm	6,56		5,49	
Ingredients				
Casein (total)	169,8		147,5	
Casein (bioavailable)	152	608	132	528
Cystine	3	12	3	12

Total Cornstarch	0	0	241	
Starch	0	0	209,7	838,68
Protein in lard	16,7	67,0	12	48
Milk fat (butter)	**340,2**		**243,9024**	
Protein from butter	1,7	6,8	1,2	4,9
Fat from butter	279	2511	200	1800
Cocoa butter	139	1422	100	900
Lard	279		200	
Fat from lard	242,73	2184,57	174	1566
Soybean oil	10	90	10	90
Cellulose	70	0	70	0
Mineral Mix	35	0	35	0
Vitamin Mix	10	40	10	40
Choline bitartrate	2	0	2	0
Total	**1058**	**6941,335**	**1062,4**	**5827,558**
Per kg		6560,335		5485,336

Tabell 2:2: Feed ingredients.

Ingredient	Supplier
Casein, purified	Altromin
Mineral premix	Altromin
Vitamin premix	Altromin
Cornstarch	Altromin
Cornstarch	Maizena
Dairy butter, salted	TINE
Cocoa butter	økosjokolade
Lard, fresh	Gilde
L-Cystine	DYETS Inc
Choline Bitartrate	DYETS Inc
Cellulose	DYETS Inc
Avicel (cellulose)	Sigma-Aldrich

3 METHODS

3.1 Experimental design

3.1.1 Experimental setup

A total of 30 transgenic NF-κB-luc mice in C57BL/J background were used in this study. They were divided into three experimental groups (n=10/group): 1) Control group, fed a control diet (CD), 2) Ketogenic diet (KD) and 3) Ketogenic diet + Fasting (KDF) (see Figure 3.1). Each group had an equal mix of age matched males (4) and females (6). All mice were fed their respective diets for three weeks until the last experimental day (day 21 or day 23) where all mice were subjected to LPS administration (1 mg/kg) to induce acute inflammation and finally terminated. One group was fed CD, consisting of 75E% fat, 15E% carbohydrates and 10E% proteins. The KD and KDF groups were both fed the same diet (Ketogenic) with 89E% fat, 1E% carbohydrates and 10E% proteins until the last day of experiment: Mice in the KFD group was fasted the last 12 hours at the end of the diet period and prior to LPS administration whereas the other two groups were fed ad libitum in the same period. Food consumption, weight and body composition were measured during the diet period. Additionally, blood glucose and ketone bodies were measured at regular intervals. On the last day, all mice were injected with LPS and subjected to *in vivo* imaging (NF-κB), termination and organ harvest. Mice were randomly assigned to two different termination days with 14-15 animals per day (15 animals on day 21, 14 animals on day 23). In advance to the final days (day 21 and 23), the mice were additionally grouped according to the *in vivo* imaging. The mice were divided into a total of 9 picturing groups (PG). Each PG contained at least 1 CD mouse, and then filling up with either KD or KDF mice. An equal number of mice from each diet groups were selected for both the final days.

See also Figure 3.2 for timeline of experiment

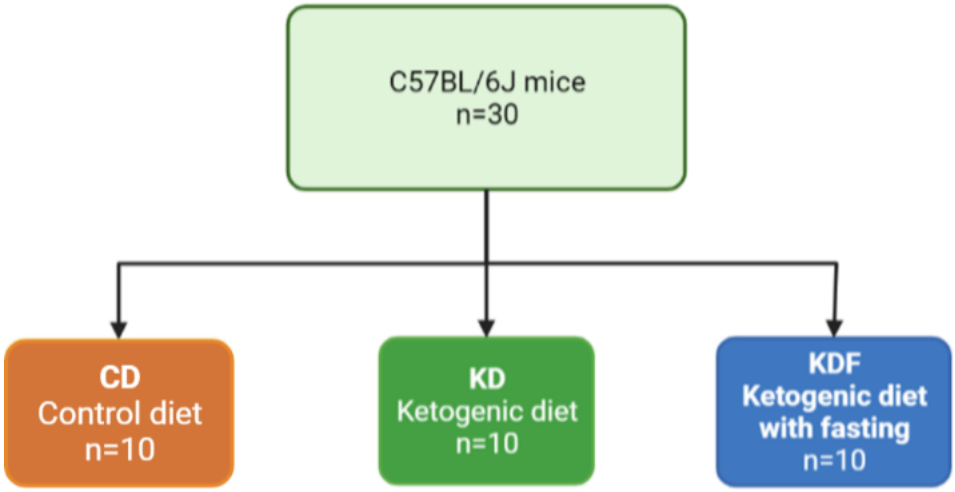

Figure 3.1: Separation of experimental group.

The mice were separated into 3 groups as illustrated in Figure 3.1. Each group consisted of 4 males and 6 females, whereas all were equally distributed in respect to age.

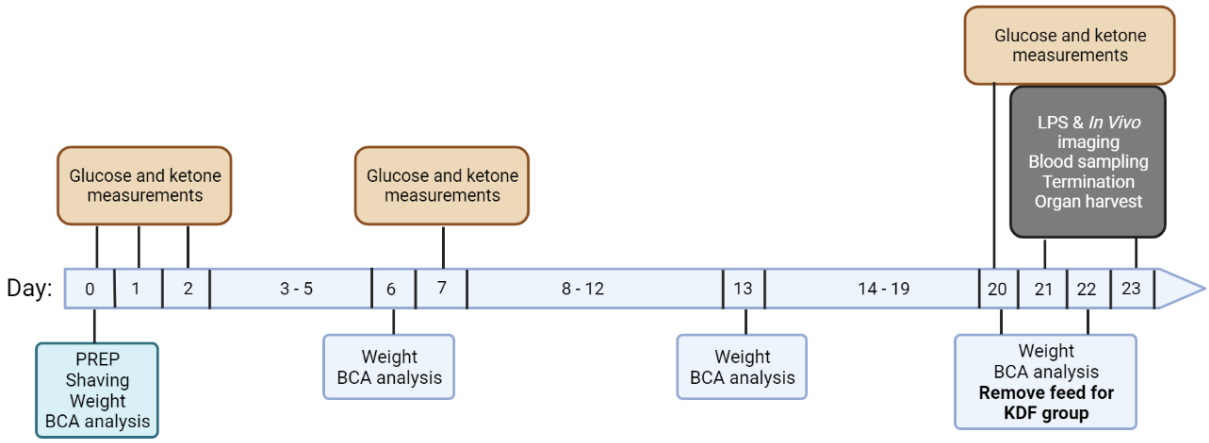

Figure 3.2: Timeline of the diet period. The mice followed their diets for a total of 3 weeks (+2 days for the second half). All control measurements were done on day 0 (pre-diet start).

In advance to the final days (day 21 and 23), the mice were additionally grouped according to the *in vivo* imaging. As the IVIS chamber used for the imaging contained 5 total spots, the mice were divided into a total of 9 picturing groups (PG). Each PG contained at least 1 CD mouse and was filled up with either KD or KDF mice (Figure 3.3). An equal number of mice from each diet groups were selected for both the final days.

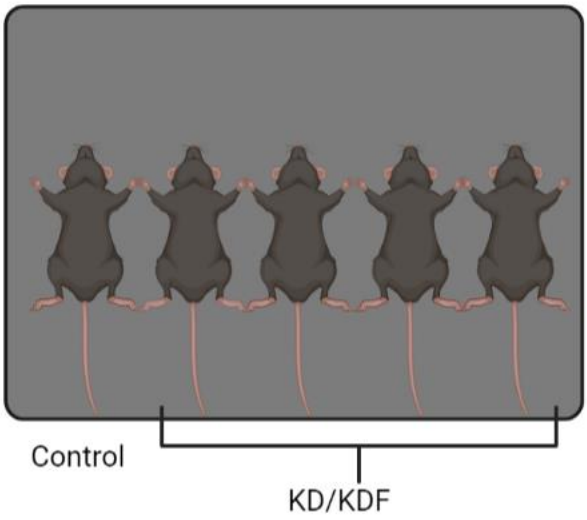

Figure 3.3: Picturing group example. Ventral side of the mice are faced towards the camera.

3.1.2 Animals and housing conditions

Heterozygous transgenic NF-κB luciferase (NF-κB-luc) mice were bred with Wild-types (WT) at the animal facility at KBM, NMBU (Figure 3.4). The estimated outcome of offspring to be NF-κB-luc was 50%. However, the result gave a total of 103 mice, in which 74 was NF-κB-luc mice. Due to this, we suspect that both parents in some of the breeds were both NF-κB-luc.

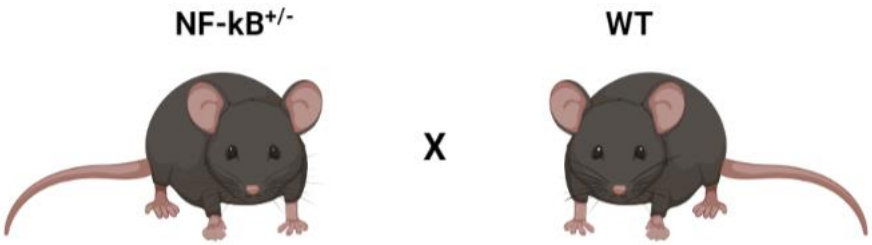

Figure 3.4: Heterozygote breed. At the animal facility, heterozygous NF-κB luciferase transgenic mice (NF-κB-luc) were bred with Wild-type (WT) mice to produce offspring, of which 50% was estimated to be transgenic.

The animals included in the experiment was a total of 30 NF-κB-luc mice C57BL/6J bred at the animal facility. Of the 30 mice, 12 were males and 18 females. The age of the mice ranged between 15-20 weeks of age at experiment start.

The mice were separated into cages of two individuals of equal gender per cage on a 12-hour light/dark cycle with available food and water *ad libitum*. During cage separation the males were co-opted with their brother to avoid conflicts of dominance. The cages were individually ventilated (Innovive, San Diego, CA). A local humidifier (Condair CP3mini, Qviller, Norway) ensured relative humidity between 45-55%. Room temperature was monitored daily and kept between 23-25°C.

3.1.3 Ethical aspects

The experiment was approved by The Norwegian Animal Research Authority (Mattilsynet). All procedures complied with EU's directives on animal research. FOTS ID for this experiment was 28945.

The two diets (KD and CD) used in this study were made in house. The KD contained, in energy 89% fat, 10% protein and about 1% carbohydrate of TCAL. The CD contained, in energy 75% fat, 10% protein and 15% carbohydrate of TCAL.

For both the KD and CD sources of fat, carbohydrates, proteins, and essential micronutrients were similar. Fat sources were fresh lard, butter, and cocoa butter. In addition, a small amount of fat came from soybean oil. Proteins came from casein (Altromin, Detmold, Germany) and carbohydrates were starch (Maizena, Fornebu, Norway and Altromin, Detmold, Germany) and a tiny amount of sucrose from vitamin mix. Vitamins and minerals were provided from a predefined Vitamin- and mineral mix (Altromin, Detmold, Germany). The ingredient list and recipe for the diets can be found in Table 2.2 (Subchapter 2.4). All dry ingredients were mixed in a bowl. Fresh lard (Gilde) from was weighed, then homogenized in a food processor. The solid fat-ingredients (dairy butter from TINE and cocoa butter from økosjokolade) were weighed and placed in a cooking pot on the stove with low-temperature. The melted fat-mix and soybean oil was added to the food processor with the lard and blended further to create a liquid fat-lard homogenized mix. No water was added.

By using a sharp knife, the tip of a 12 mL syringe was cut off. The syringe was used to load portion-sized, rod-formed feed-pellets. The feed-pellets were pushed onto a baking paper-covered baking sheet. When all the feed-pellets were placed onto baking sheets, the baking sheets were cooled in a fridge at approximately 4℃. After cooling for an hour, the pellets were packed in food vacuum packaging bags and marking each with either "Keto" or

"Control". The bags were sealed with a vacuum machine by using the half-vacuum option. The packed feed was stored in a freezer at -20°C.

3.1.4 Optimization of feed

The high content of fat in both CD and KD made the diet pellets soft. To test if the feed would remain stable and firm in the food trays in each animal cage, we placed the pellets in the food tray of an empty cage inside the animal lab. We then did sensory analyses of the feed. After 1 week, no drip from the feed through the dispenser was observed, nor did it show any signs of foul smells or oxidation. After 2 weeks, the feed had a foul smell to it, but was still firm. From this test, we decided to replace the feed every 4-7th day during the experimental period.

We tested if the mice would eat the KD by feeding it to a selection of C57BL6/J mice not enrolled in the study. The weight of the feed was measured before and after the test and divided by the number of mice (n=4) to control if the average daily consumption per mice would comply with their caloric needs. The average consumption per mice from this test was calculated to comply with the caloric needs of the mice (no data).

3.2 Ketone body measurements

Due to our desire to monitor state of ketosis during the experimental period, a handheld ketometer tool for rapid and consecutive measurements was of interest. The ketometer Freestyle Precision Neo (FPN) from Abbott measures BHB and utilizes strips that requires a tiny sample of blood (~2-3 µl) per measurement and shows the result within seconds. However, how accurate and precise FPN was for usage for mice blood was unclear to us. With an assay kit from MegaZyme (originally designed for BHB quantification in food products) that we had ordered earlier, we decided to compare the values from FPN with MegaZyme. However, since MegaZyme required plasma samples for BHB quantification, we also decided to compare blood BHB to plasma BHB with FPN after centrifuging the blood samples to plasma.

3.2.1 MegaZyme BHB Assay kit

Three samples of blood were spiked with a BHB-salt (*DL-β-hydroxybutyric acid sodium salt*, Sigma-Aldrich, St. Louis, MI) to achieve different known concentrations. The samples were then analyzed and compared with FPN and MegaZyme.

From the result of this sub experiment, we concluded that measurements of BHB concentration in whole blood were more accurate when measured with FPN compared to plasma, and that FPN was more suitable than MegaZyme for measuring BHB in mice blood in terms of accuracy.

3.2.2 Weight and body composition analysis

Time-domain nuclear magnetic resonance (TD-NMR) is a technology based on the capture of radiofrequency signals from hydrogen spins from fluid and soft tissues. TD-NMR Minispec LF50 (Bruker, Billerica, MA, USA) utilizes contrasts of relaxation times of hydrogen spins from liquid, fat, and lean mass to estimate body composition (BC) (Morla et al., 2020). The non-invasive method makes this technique favorable for consecutive measurements of BC in mice. By determining these bodily values, the health state of the mice could be assessed during the experimental period while also comparing the values to account for any significant differences of the final biomarker analyses.

Mice were weighed and analyzed for BC including bodyfat and lean mass once per week. TD-NMR Minispec LF50 (Minispec LF50) was calibrated for mice in the weight range of 5g to 60g. Magnet temperature was set to 37℃ and proton frequency was 7.5 MHz. A daily check was required, where a standard sample of chia seeds with a known amount of fat was inserted into Minispec LF50 and the "Daily check" command was run. The mouse was picked up by their tail and weighed inside a plastic container on a digital scale. The mouse was further transferred into a red, transparent plastic restrainer that was tilted horizontally to allow the mouse to move to the bottom by itself. A plastic piston was inserted into the restrainer to fixate the position of the mouse to the bottom and limit its mobility. The plastic restrainer was inserted into the Minispec LF50 and measurements were acquired. The mouse was returned to its cage and the restrainer was cleaned with water.

3.2.3 Energy intake

Feed trays, as well as any remaining feed particles (RFP) in the cage were weighed every 4th to 6th day to assess feed consumption. Feed intake per mouse was estimated by calculating the delta-value of the feed tray, including any RFP and dividing by the number of mice per cage (n = 2). Feed trays were also refilled the same day. The formula for calculating the delta tray-weight value is illustrated in **(2)** below:

$$\textbf{ΔTray weight} = Newly\ filled\ tray - (Tray\ weight\ at\ day\ X + \ RFP) \quad \textbf{(2)}$$

Day X indicates the day the feed was switched out and weighed. Abbreviations: RFP =Remaining feed particles

Whereas the total consumption per mice is calculated as shown in formula **(3)** below.

$$\text{Total consumption per mice} = \frac{\Sigma\ (\Delta\text{Tray weight})n}{n\ mice} \quad \textbf{(3)}$$

The number of mice within each cage is shown as n mice (n = 2).

3.2.4 Blood glucose and state of ketosis

Blood samples for non-fasting glucose- and ketone levels were measured the day before diet start, and then the following two first days during the diet period. The blood samples were taken by shaving the left leg to the mouse and provoking a blood droplet by punching a hole in the leg vein with a 25G syringe-needle. The purpose of the first three days of measurements was to assess the time required to reach nutritional ketosis in the animals by observing the dynamics of ketone levels. To monitor the glucose and ketone levels throughout the rest of the experiment, samples were taken after one week, three weeks and within the final hour before LPS injection. The blood glucose measurements were performed with a blood glucose meter and test-strips from Accu-Chek (Roche Diagnostics). Blood ketones measurements were performed with a blood ketone meter and test-strips from Freestyle Precision Neo (Abbott).

3.2.5 Initiating fasting

To evaluate the effects of fasting after being fed KD for 3 weeks, the feed and any RFP to the ketogenic/fasting group (KDF) was removed the evening prior to the last day (8 hours prior to LPS injections).

3.3 Analysis of NF-κB actvitiy

The animals were subjected to an acute inflammatory response by LPS (*Escherichia coli* serotype 055:B5, Sigma-Aldrich) and analyzed for NF-κB activity to determine whether the diets influenced the inflammatory response differently. We initially wanted to do 2 hour and 4 hours past LPS *in vivo* analyses. Due to blood sampling and dissection part being more time consuming than we anticipated, the *in vivo* analysis was performed at 2 hours and 5 hours past LPS injection.

3.3.1 LPS injections

To create an acute inflammatory response, mice were injected intraperitoneally with 100 µl lipopolysaccharide (LPS) per 25-gram mouse (1 mg/kg bw). The doses were calculated according to the weight of the mice measured earlier the same day. A 1 ml syringe with a 25G needle was used for the LPS injections.

3.3.2 IVIS imaging of NF-κB transgenic mice

Bioluminescence imaging is a methodology developed as a tool for molecular imaging. IVIS Lumina II (IVIS, Lumina II, PerkinElmer, MA, USA) is a quantitative and bioluminescent imaging instrument designed for *in vivo* analyses of small laboratory animals. The instrument includes a highly sensitive CCD camera, light-tight imaging chamber and 5 gas anesthesia chamber inlets, which allows multiple *in vivo* analyses to be performed at once. The CCD camera is thermoelectrically cooled to -90℃, ensuring low background noise. The CCD camera detects visible light produced from luciferase during luciferase-mediated oxidation of luciferin, at a wavelength of 560 nm (Zhu et al., 2017).

Mice were anesthetized by transferring them into the isoflurane chamber and turning on the isoflurane supply. During anesthesia, the thorax-abdominal area of each mouse was shaved. 1 hour and 45 minutes past baseline LPS injection, the mice were injected with luciferin (200 µl/25g mouse) with the same one-minute interval as the LPS injections. The mice were placed into the IVIS chamber on their backs with their snouts pointed into the anesthesia inlets to secure anesthesia during the imaging process. Imaging period was set to 60 seconds. 10 minutes after luciferin injections imaging was acquired. Mice were then returned to their cages. Luciferin injections and IVIS analysis was repeated after 4 hours and 45 minutes. This time after imaging the mice was not returned to their cages but remained in anesthesia inside

the IVIS chamber for blood sampling. The mice were consecutively operated for the next step.

3.4 Blood sampling

Approximately 400-800 µl of blood was required for further cytokine and biomarker analyses and was collected from the heart by cardiac puncture. Syringes were filled with (50-100 µl) EDTA, and blood samples were taken while the mice were still anesthetized inside the IVIS chamber. This was done to enhance the efficiency of the sampling process. Blood samples were transferred to 1,5 ml Eppendorf tubes and put on ice. After blood samples from the PG were collected, the mice were euthanized through cervical dislocation. The blood samples were later centrifuged to obtain plasma.

3.5 Organ sampling

To acquire samples from organs for the *ex vivo* analysis, euthanized mice were fixated on an aluminum-covered Styrofoam plate and dissected. Intestines, liver, and brain were surgically removed and systematically placed onto a black paper sheet. The sheet with organs for the *ex vivo* analysis was placed into the IVIS chamber and imaged for 5 minutes. The next group of mice was now ready for the 5-hour *in vivo* imaging, blood sampling and organ harvest.

3.6 Cytokine profiling with ProcartaPlex

ProcartaPlex Immunoassays (Invitrogen, ThermoFischer) is a magnetic microsphere technology developed for multiple protein detection and quantification in several biological matrices. The platform allows detection of multiple cytokines from a single sample, making it useful for assessing cytokine profiling. This immunoassay was used for the frozen plasma samples acquired earlier, to quantify the cytokines TNFα, IL-6, IL-1β, IL-10, IL-18 and IFN-γ with Bioplex-200 systems (Bio-Rad).

3.6.1 Magnetic microspheres

The ProcartaPlex kit is supplied with magnetic beads that are coated with analyte-specific capture antibodies. The capture antibodies bind to a specific analyte, which in this experiment is a cytokine. A biotinylated detection antibody (DA) which recognizes the analyte and binds to it is then added and forms an antibody-cytokine-antibody sandwich. A further addition of

phycoerythrin-streptavidin (SAPE), which binds to the biotin on the DA completes the microsphere complex. Phycoerythrin emits fluorescence. The fluorescence property together with each unique beads, enables the classification and quantification of the different cytokines with a flow-cytometry-based platform. The magnetic beads are retained during washing processes, by using a magnetic plate holder (Houser, 2012). An illustration of the principle of magnetic microsphere technology is shown in Figure 3.5 below.

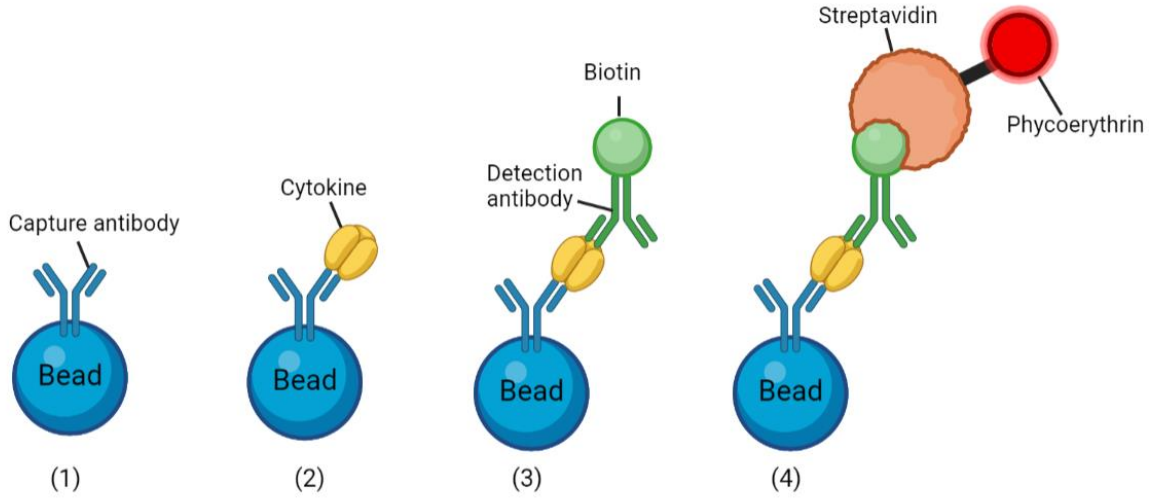

Figure 3.5: Principle of magnetic microsphere technology. The types of beads are distinguished by color, here one type is shown in blue, and is modified with an analyte-specific capture antibody **(1)**. The capture antibody binds to the specific cytokine **(2)**. A biotinylated DA recognizes the specific cytokine and binds to it **(3)**. Upon addition of SAPE, streptavidin binds to biotin with high affinity **(4)**. The figure is created in Biorender.com.

3.6.2 Bio-Plex 200 systems

The ProcartaPlex assay kits are compatible with Bio-Plex 200 systems. Bio-Plex 200 systems utilizes the principles of flow-cytometry that enables multiple analytes from small sample sizes to be analyzed. The distinctly colored beads allow simultaneous detection of multiple analytes in the same sample. Bio-Plex is combined of a fluidics system, two lasers and digital signal processors. The fluidics system ensures the beads are aligned and pushed through a detection chamber with two lasers in it. One of the lasers is a classification laser that recognizes the dye color in each bead and identifies the beads. The other laser excites fluorescence from phycoerythrin associated with the bead, which allows quantification of the analytes. Digital signal processors record the fluorescent signals simultaneously for each bead and translates the information into data for each bead-based assay (Houser, 2012). The

principle behind classification and quantification of beads with Bio-Plex 200 is illustrated in Figure 3.6.

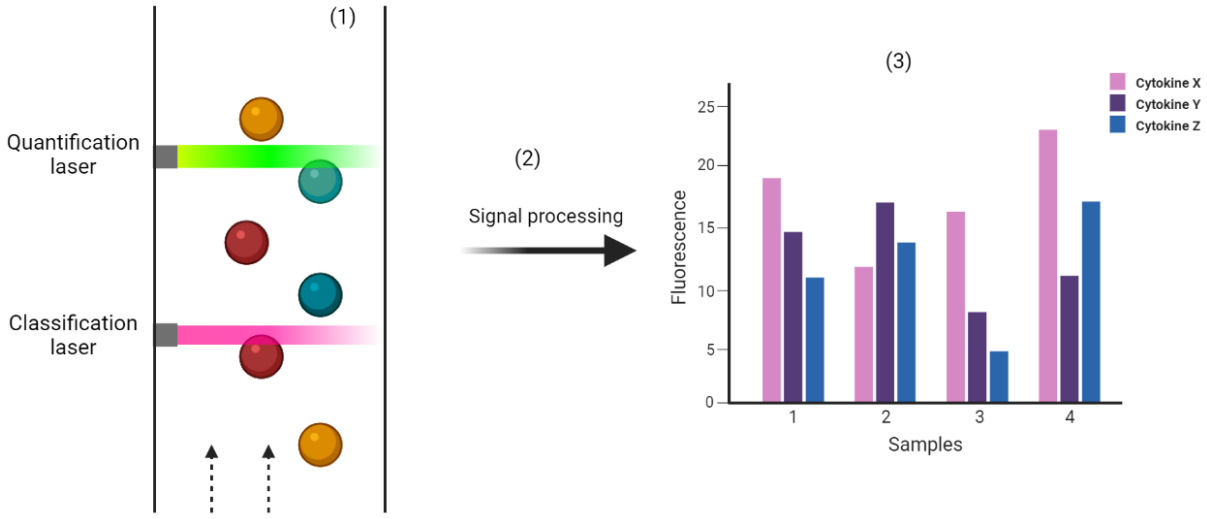

Figure 3.6: Bio-Plex 200 systems bead detection principle. Beads are aligned into a detection chamber with two lasers; a classification laser that identifies the beads by their color and a quantification laser that recognizes the fluorescence from the reporter molecule phycoerythrin **(1)**. The signals are transduced by digital signal processors **(2)**, to receive data from each sample **(3)**. The figure is created in Biorender.com.

3.7 Statistical analyses

Normal distribution was tested by using Shapiro-Wilks test. Comparisons between groups were done by using analysis of variance (ANOVA) and Tukey tests. Interaction terms were included in the ANOVA analyses, but due to no significant interaction terms ($p < 0.05$) in any of the analyses, the models were simplified without interactions. If a significant gender effect was observed, the results are shown in separate figures, stratified by gender, if not otherwise specified. To test for differences between measurement days within groups, a paired T-test was performed. Upon non-normal distributed data, the original values were transformed to logarithmic values (log10) to fit normal distribution. Comparisons within or between the groups were considered statistically significant when p-value were < 0.05.

4 RESULTS

4.1 Characterization of body weight, body composition and feed intake

To monitor and determine weight changes during the experiment, the animals were weighed every week. As shown in Figure 4.1-A, the average baseline weight differs somewhat amongst the groups. During the 3-week feeding period, minimal changes in total body weights were observed and no significant difference in weight gain was found between the groups. However, a gender effect ($p = 0.0305$) was observed, suggesting that females generally gained slightly more weight than males.

We next investigated if body composition was affected by the diets by assessing fat mass and lean mass using TD-NMR Minispec LF50. Since KD and KDF followed the same diet during the period the two groups were merged to one. All groups gained bodyfat from Day 0 to Day 20 ($p = 10^{-5}$). After 20 days, CD had a significantly higher bodyfat percentage compared to KD/KDF ($p = 0.0351$). No significant change in lean mass was observed.

Additionally, feed intake was measured at 4 to 7-day intervals through the experiment and total energy intake per mouse was calculated by adding the delta values of feed weight (see formula **2** and **3)**. No differences between the groups were observed in energy intake (Figure 4.1.E). A quick calculation shows that the total energy intake equals a daily energy intake of 12-14 Kcals per mouse, which complies to the required energy intake of laboratory mice (National Research Council Subcommittee on Laboratory Animal, 1995).

Since the diets were homemade, we were concerned if mice would tolerate and eat the feed. Prior to the experiment, this was tested, and intake of the feed was deemed adequate. However, we observed that two KD/KDF mice lost weight during the first week. One of them looked quite ill and was euthanized. The other lost weight during the two first weeks, but the behavior and health seemed otherwise fine. This mouse was monitored daily and eventually regained its weight and was included in subsequent analyses.

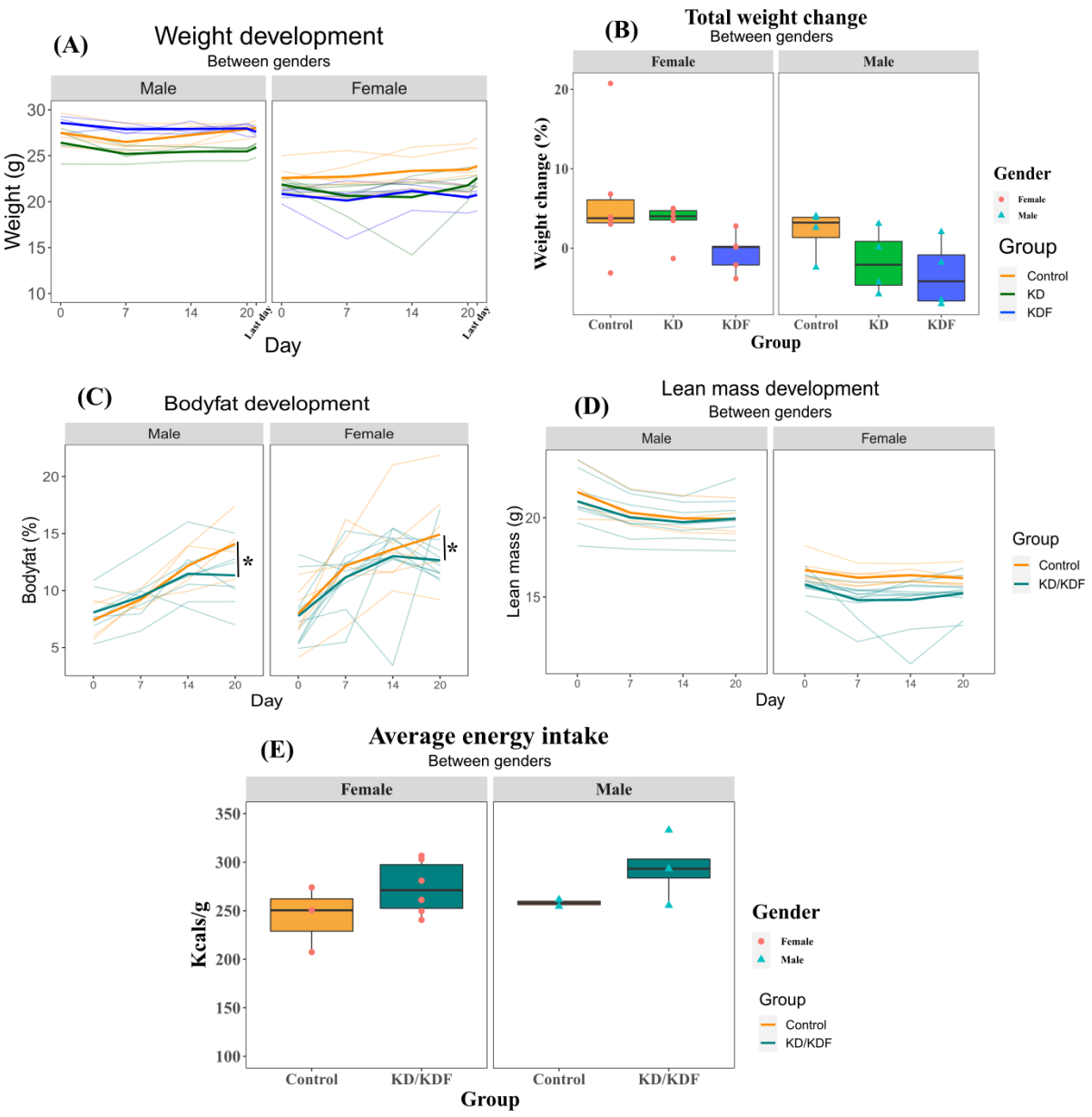

Figure 4.1: Weight, body composition and energy intake. The figure shows the development of weight **(A)**, total weight changes **(B)**, development of bodyfat percentage **(C)**, lean mass **(D)** and energy intake **(E)**. Genders in bodyfat percentage **(C)** are shown separately. **(A), (C)** and **(D)**: Individual datapoints are shown as transparent colored lines with colors matching the group of the individual, while opaque colored lines illustrate the average values within the groups. **(C)**: Significant differences between the groups are shown as * for $p < 0.05$. For the boxplots, the horizontal line through the box represents the median value. The box represents the interval in which 50% of the data resides, whereas 25% of the data is above and 25% of the data is below the median within the box. Whiskers extend to the largest and lowest value, excluding outliers. Outliers are plotted as dots beyond the whiskers.

4.2 Blood ketones and glucose levels

To monitor BHB development and non-fasting blood glucose development in the animals, BHB and blood glucose were measured simultaneously. We hypothesized that BHB in KD and KDF would reach peak values within a few days, so we wanted to do four days of consecutive measurements. Due to unpredictable delivery of ketone strips, we were unable to do measurements on Day 3. Further measurements were done on Day 7, 20 and the Last day. Figure 4.2-A shows the dynamics of blood BHB concentration between the groups and genders during the experiment. A gender effect was observed on Day 7 ($p = 0.0449$), Day 20 ($p = 0.0074$) and Last day ($p = 0.0052$), indicating that females maintained higher BHB levels at these days compared to males. At Day 2, KD and KDF were higher than CD ($p = 0.0000$). At Last day, KD were higher than CD ($p = 0.0014$) and KDF were higher than CD ($p = 10^{-5}$).

Additionally, to test for differences in BHB concentration between Day 2 and Day 20, a paired T-test was performed for KD and KDF, both genders combined. The T-test revealed a significant decrease in BHB concentration between Day 2 and Day 20 for KD/KDF combined ($p = 0.0001$).

In summary, these results show that mice enter ketosis within 1-2 days of KD feeding, while fasting after being fed a KD for 3 weeks increases blood BHB concentration further. Females maintains higher blood BHB levels than males. Additionally, BHB concentration drops after peaking, but remains higher than CD.

Female non-fasting blood glucose levels in Figure 4.2-B were lower than males at Day 20 ($p = 0.0026$). Baseline (Day 0) non-fasting blood glucose levels were surprisingly different for KD ($p = 10^{-5}$) and for KDF ($p = 0.0002$) compared to CD. Regardless, the graphs behave in an almost mirrored manner in comparison to Figure 4.2-A. At Day 2, KD and KDF were both lower than CD ($p = 10^{-5}$). At Last day, KDF were different than both KD ($p = 0.0092$) and CD ($p = 0.0077$). However, KD were not different than CD at Last day.

To test for differences between Day 2 and Day 20, a paired T-test was used on Day 2 vs Day 20 for the KD and KDF combined. The T-test revealed an increase in blood glucose between Day 2 and Day 20 for KD/KDF ($p = 0.0055$).

Our results show that a sudden drop in non-fasting blood glucose levels occurs within 1-2 days in mice switching towards a KD but increases and seemingly stabilizes after a week. After fasting for 8 hours, blood glucose drops further.

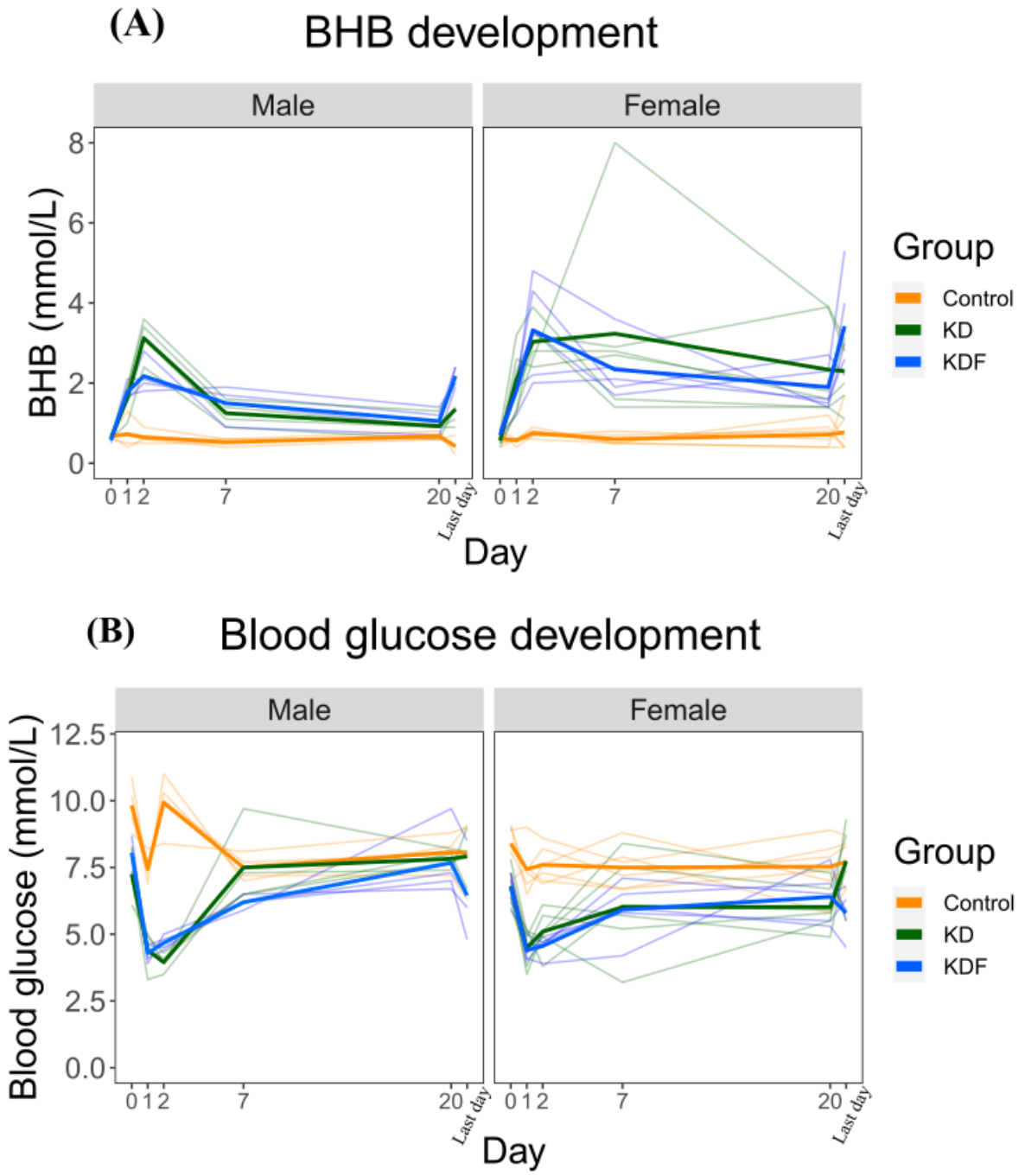

Figure 4.2: Blood BHB and glucose development. (A) BHB blood concentration and **(B)** non-fasting blood glucose concentration. Measurements were taken on the first three days, then Day 7, 20, 21 and 23. Measurements from Day 21 and 23 is here combined and shown as Last day. Feed was removed from KDF group 8 hours in advance to LPS injection to the last day. Individual datapoints are shown as transparent colored lines, with colors matching the group of the individual. Opaque colored lines illustrate the average values within the groups.

4.3 *In vivo* analysis of NF-κB activity over time

To investigate how the diets affected NF-κB activity after LPS-induced inflammation in the animals, we performed *in vivo* imaging of NF-κB activity at 2 hours and 5 hours following LPS injection (1 mg/kg). Following imaging acquisition (60 sec exposure time), images were processed and assessed for NF-κB mediated light intensity in the thoracic-abdominal area. NF-κB activity was increased at 5h compared to 2h ($p = 0.0098$). A possible reduction in NF-κB activity was observed for KD compared to CD with and for 2h ($p = 0.1095$) and 5h ($p = 0.1259$). The results here indicate that NF-κB activity increases ($p = 0.0098$) from 2 hour to 5 hours following LPS injection, with a possible reduction in NF-κB activity for KD compared to CD.

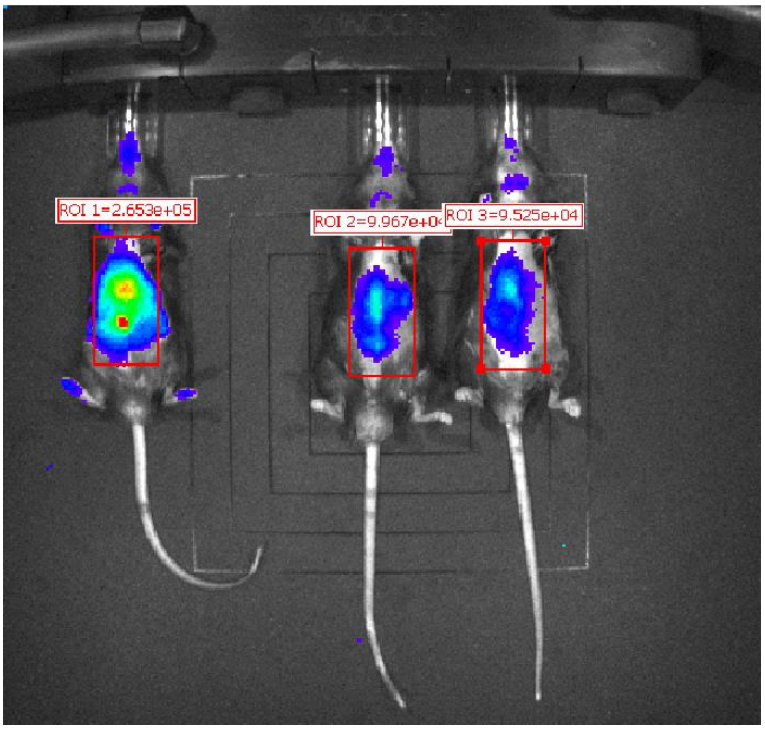

Figure 4.3: In vivo ROI selection. The region of interest (ROI) was selected by using a square outline tool on the thoracic-abdominal area of the animal, using its elbow joint and inner thighs as reference points. Here, a CD mouse (to the left) shows a stronger light emission than KD mice (middle and right).

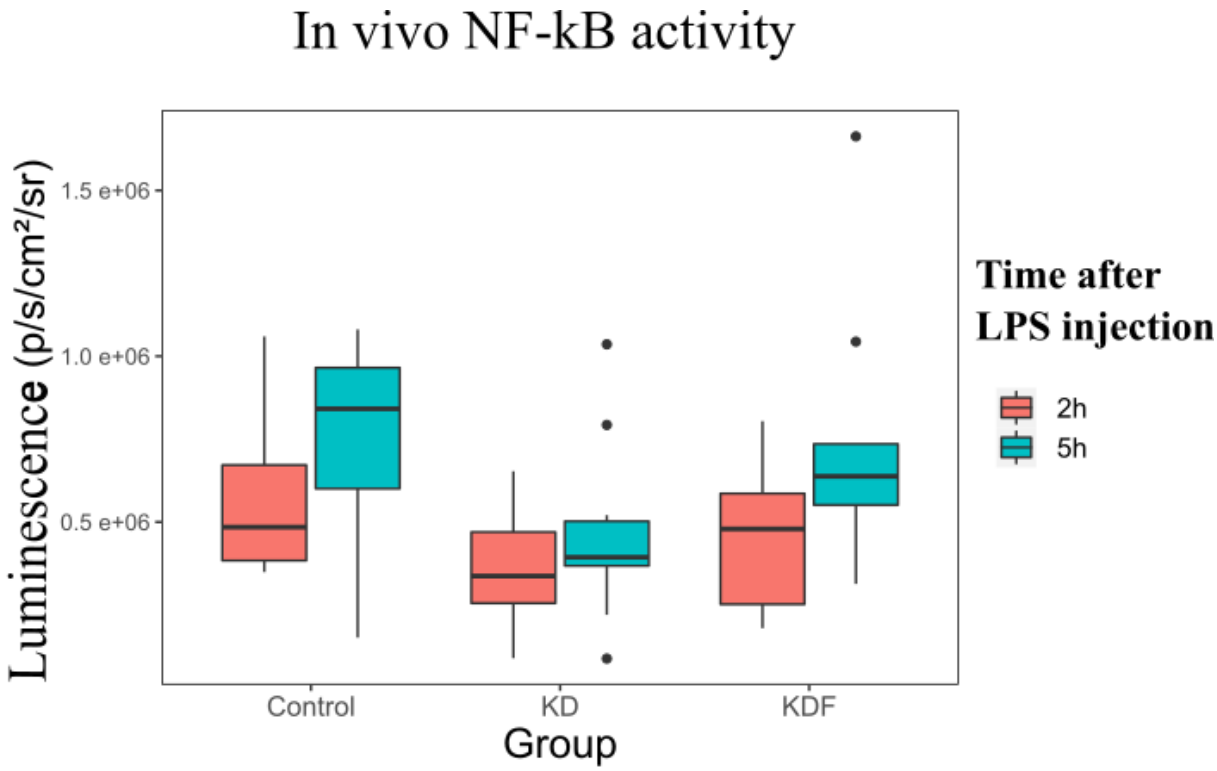

Figure 4.4: In vivo NF-κB activity. The figure shows luminescence (p/s/cm^2/sr) between the groups at 2 and 5 hours past LPS injection. The horizontal line through the box represents the median value. The box represents the interval in which 50% of the data resides, whereas 25% of the data is above and 25% of the data is below the median within the box. Whiskers extend to the largest and lowest value, excluding outliers. Outliers are plotted as dots beyond the whiskers.

4.4 *Ex vivo* analysis of NF-κB activity

We next investigated single organs for NF-κB activity by *ex vivo* imaging. NF-κB activity in liver were generally higher in males compared to females ($p = 0.0007$). A possible gender interaction was observed in liver NF-κB activity ($p = 0.0567$), suggesting that males may have reacted differently than females to both diets. A possible trend of reduction in liver NF-κB activity amongst KD compared to CD was observed ($p = 0.0637$).

Since the organ harvesting process was somewhat time consuming (2-3 minutes per mouse), we decided to set the imaging period to 5 minutes to compensate for possible oxidation and loss of luciferase activity. During organ harvest of the mice, we noticed that in total 4 of the KD mice had developed a moderate degree of fatty liver. Additionally, one of the CD mice had a state of hyperplasia in the ovary, but the size of the ovary indicated that the organ defect

may have progressed over several weeks, likely long before experiment start. *Ex vivo* analysis of the different organs is illustrated in Figure 4.5 below.

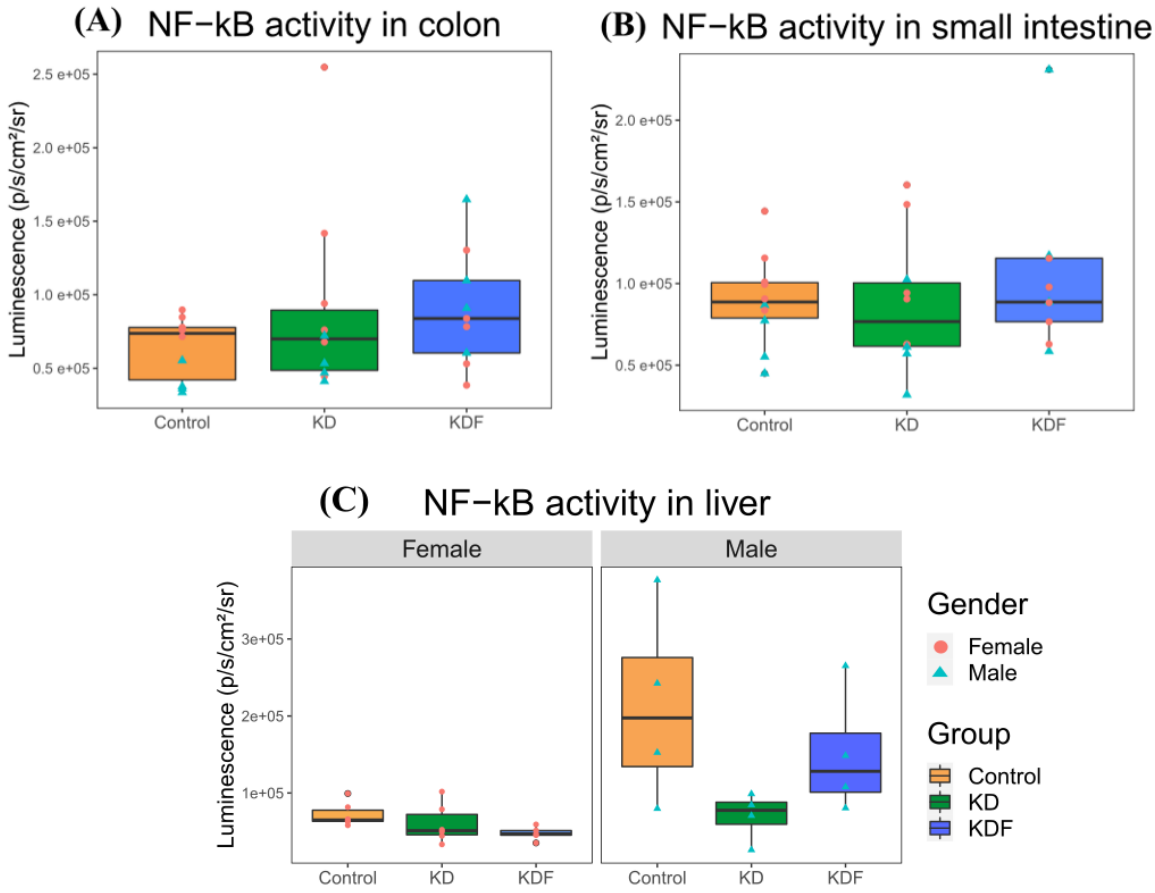

Figure 4.5: Ex vivo radiance. Luminescence from **(A)** small intestine, **(B)** colon and **(C)** liver. Analyses were performed with IVIS Lumina II. The horizontal line through the box represents the median value. The box represents the interval in which 50% of the data resides, whereas 25% of the data is above and 25% of the data is below the median within the box. Whiskers extend to the largest and lowest value, excluding outliers. Outliers are plotted as dots beyond the whiskers.

4.5 Correlation of NF-κB activity between *in vivo* and *ex vivo* analysis

A dominant proportion of the *in vivo* radiance seemed to originate from the liver area. Due to this, we investigated the connection between *in vivo* and liver *ex vivo* radiance. Figure 4.6 illustrates the linear correlation between *in vivo* radiance and *ex vivo* liver radiance. A

moderate correlation with R = 0.59 was observed, suggesting a moderate-high connection between the two variables.

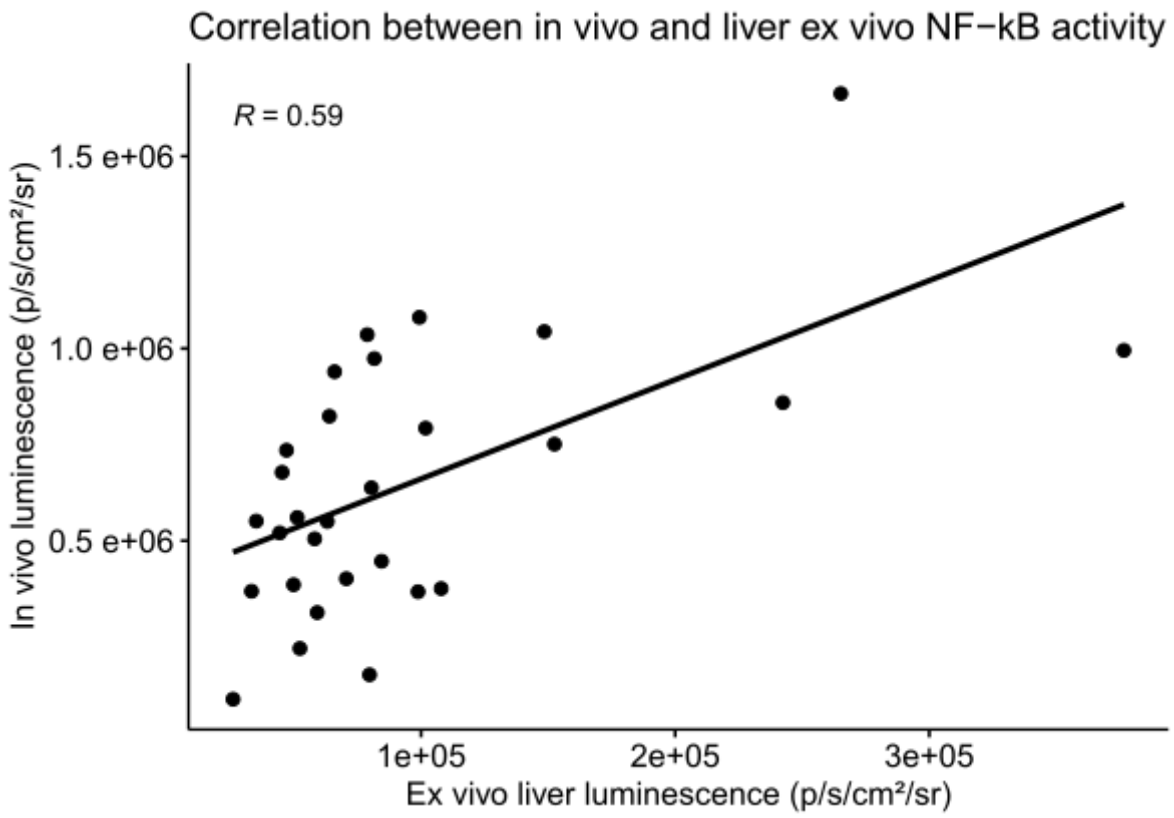

Figure 4.6: In vivo and ex vivo NF-κB activity. The figure illustrates the correlation between in vivo (y-axis) and liver ex vivo (x-axis) NF-κB activity. Pearson correlation coefficient (shown as R) analysis was performed to investigate the linear correlation between the two variables.

4.6 Cytokine profiling

To investigate how the diets affected the production of cytokines in the animals, we analyzed the plasma samples for cytokine profiles. After running the 96-well plate once, several errors occurred in most samples. The errors (1, 2, 3, 4, see manual for BioPlex-200 in Appendix - D) included undetectable bead numbers, bead classification issues, region assignment issues and bead coagulation. Shortly after, we contacted one of BioRad's technical support who suggested switching out the sample needle and run the 96-well plate once more after washing the plate. This time we were able to receive usable data for most cytokines except for IL-18, which was not detectable in any of the samples. Figure 4.7 illustrates the different cytokine plasma concentrations. Male IL-6 concentrations (Figure 4.7-E) were generally higher than females ($p = 0.0045$). TNF-α (Figure 4.7-D) were higher in KDF compared to KD ($p =$

0.0071) and higher than CD, but not significantly (p = 0.0766). For IL-6, KD group had lower values than KDF (0.0115). No significant differences between the groups or genders for IL-1β, IL-10 or IFN-γ were observed.

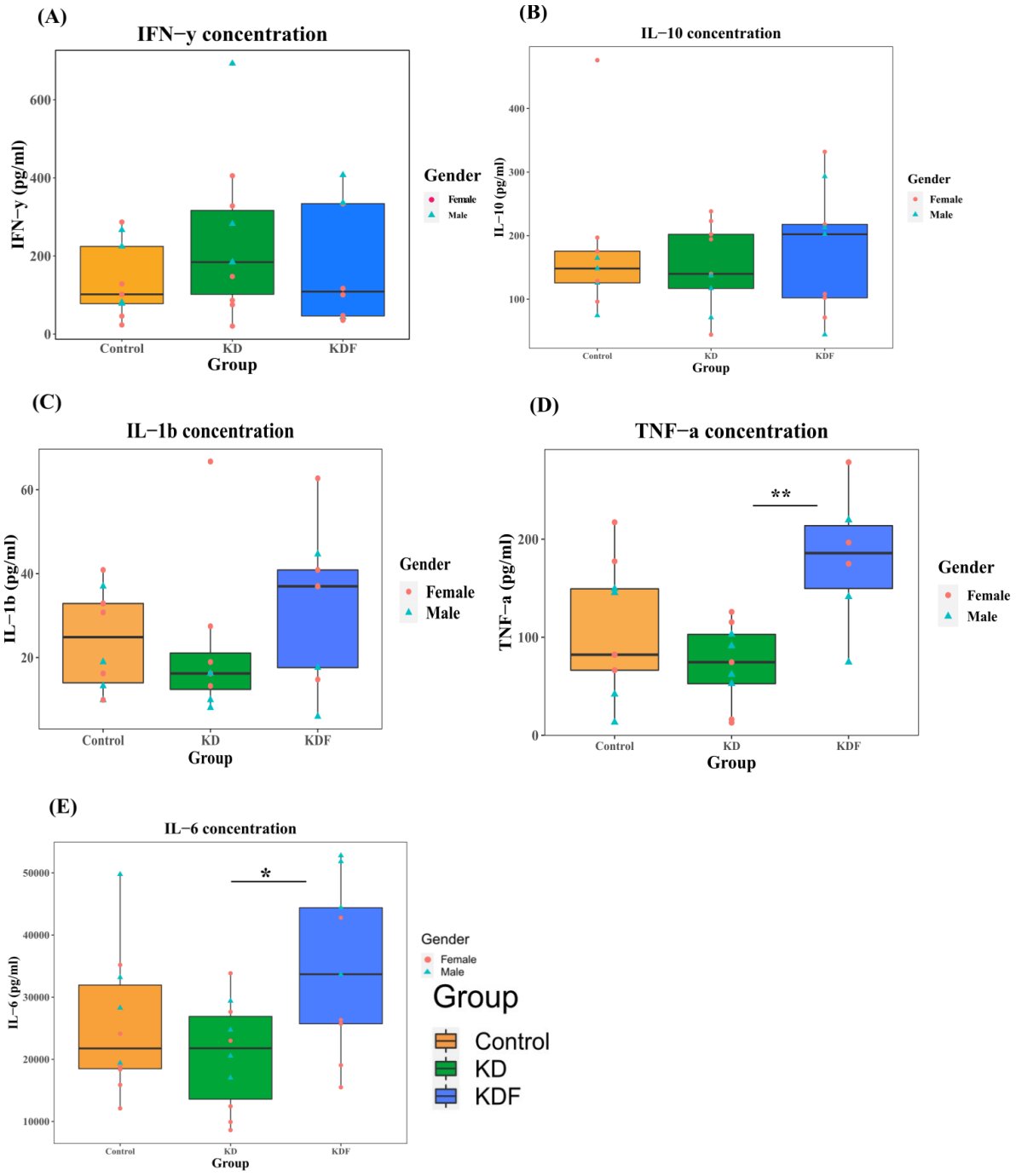

Figure 4.7: Cytokine profiling. Cytokine plasma concentration (pg/ml) of **(A)** IFN-γ, **(B)** IL-10, **(C)** IL-1β, **(D)** TNF-α and **(E)** IL-6 quantified with Bioplex-200 systems. Significant differences between the groups are shown as * for $p < 0.05$ and ** for $p < 0.01$. Number of observations within the groups were only equal for IL-6 as the others had some missing data. The horizontal line through the box represents the median value. The box represents the interval in which 50% of the data resides, whereas 25% of the data is above and 25% of the data is below the median within the box. Whiskers extend to the largest and lowest value, excluding outliers. Outliers are plotted as dots beyond the whiskers.

5 DISCUSSION

Chronic inflammation causes a physiological state of constant degradation and repair in tissues. This dysregulated inflammatory state is associated with many chronic diseases, such as cancer, diabetes, autoimmunity, and neurodegenerative diseases.

The ketone body BHB has been demonstrated to affect inflammatory markers in animal studies, and mechanistically affect mediators involved in the assembly of NLRP3 inflammasome. In human studies, implementation of KD in patients with certain chronic inflammatory diseases has been associated with a decrease in systemic inflammation (Bahr et al., 2020; Gross et al., 2019; Myette-Côté et al., 2018).

We therefore aimed in this study to test how KD with and without fasting would affect an LPS-induced inflammatory response in mice. Our main results were that genders reacted differently to the KD, in terms of blood BHB and glucose levels, liver NF-κB activity and IL-6. KD, but not KDF, mitigated TNF-α levels in both genders, and further mitigated IL-6 and liver NF-κB activity for males.

5.1 Consumption of Ketogenic and Control diets

Overconsumption or underconsumption of nutrition may impact several aspects of health regarding inflammation (Fernández-Sánchez et al., 2011; John et al., 2017). By monitoring the energy intake of the mice, we could adjust for any potential effects on the inflammatory markers that were assessed.

After a few days into the experiment, we noticed that the fur of the KD/KDF mice became quite oily. Even though the feed did not directly drip from the tray, we suspect that some of the fat from the KD was transferred from the feed tray to the mice as they were licking their fur, or as they were moving underneath the tray, possibly causing fatty particles from the feed to attach to their body. We suspect that some of the fatty particles were further transferred from the oily fur onto the ornaments or wooden chips in the cage, especially since we observed some fatty particles on the running wheel and the paper material. During feed intake measurements we tried to include all RFP we could spot in the cage. However, as smaller RFP may have been scattered throughout the cage, we believe the total feed intake was not completely accurate resulting in a greater calculated feed intake than the actual feed intake for

KD/KDF. This did not seem to be an issue for the CD. Regardless, from our calculations on feed intake, we do not believe there was a significant difference between any of the groups.

5.2 Characterization of weight, body composition and energy intake

A consensus on energy intake and weight change is that an energy intake greater than the energy expenditure equals weight gain (Howell & Kones, 2017). Fatty acids are more energy dense than carbohydrates and protein, yielding 9 kcal/g compared to the 4 kcal/g from carbohydrates and protein (Akoh, 1995). It is thus reasonable to believe that a calorie-dense, high-fat diet would increase weight gain significantly more in comparison to e.g., a low-fat diet. Besides, high-fat diets are commonly used in mice studies to induce obesity and weight gain (Yoshizaki et al., 2020). So, how did this consensus comply with our results from the 90E%-fat KD and 75E%-fat CD? Over a 3-week experimental period, no significant changes were observed in weight nor lean mass. It is likely that the 3-week experimental duration was not sufficient to show changes in weight. In the study on KD and gut microbiota by Ang et al., mice were fed similar KD and CD over 3-week periods. The corresponding KD and CD mice in that study did not differ from each other in terms of weight gain but did gain more weight than chow-fed mice. It is worth mentioning that Ang and colleagues used custom diets ordered from Envigo™. Our house-made diets may have had food components in it that were less appealing to the mice, or somehow prevented overeating. However, bodyfat still increased in all groups.

5.3 Blood BHB- and glucose levels

Limited availability in blood glucose leads to an increase in ketone body production. The circumstances that decrease glucose availability may be intense exercise, fasting or adherence to a KD. However, the physiological glucose levels will never be completely depleted as the brain is always dependent on glucose supply (López-Gambero et al., 2019). Under glucose-depriving circumstances, the maintenance of blood glucose homeostasis is regulated by gluconeogenesis, which utilizes substrates such as lactate, glycerol and glucogenic amino acids to produce glucose (Schutz, 2011).

We wanted to investigate the dynamics of the ketone body BHB during adherence to a KD and how BHB levels behaves by reducing the glucose-availability further with fasting. Based on our measurements, we found a significant increase in BHB by adherence to a KD after just

one day, with a further increase on Day 2. However, the BHB levels did not stay at those high levels throughout the three weeks but dropped and stabilized at around 1 mmol/L for males and 2 mmol/L for females.

An upregulation of the ketogenic enzymes BDH and HMGCS2 in rodents fed a HFD or KD have been demonstrated in several studies (Cullingford et al., 2002; Liśkiewicz et al., 2021; Sikder et al., 2018). Liskiewicz and her colleagues also found a significant upregulation of the ketolytic enzyme SCOT in the hippocampus and cortex in rats after feeding a KD, suggesting an increased efficiency in ketone body catabolism (Liśkiewicz et al., 2021). Besides, as demonstrated by Appleton and DeVivo, the rats gained maximum benefits of reducing seizures after "adapting" to a KD over 20 days (Appleton & DeVivo, 1974). Moreover, Bough et al. found an increase in hippocampal mitochondria in rats following a KD over three weeks.

Taking the findings from these studies into consideration, it is reasonable to believe that the upregulation of ketogenic enzymes and mitochondrial biogenesis during prolonged adherence to a KD helps maintain a "stable" state of ketosis over time, as observed from our results.

An interesting finding from the state of ketosis between the genders were observed, where females seemingly maintained higher blood BHB levels. Jikumaru et al. previously reported that female mice survived longer than male mice under starvation conditions (Jikumaru et al., 2007). The researchers stated that "The generation of ketone bodies was enhanced in female compared to male animals". By removing the ovaries from female mice, the researchers observed that the increase in ketogenesis were inhibited, while treating male mice with the estrogen steroid hormone, estradiol, increased ketogenesis. They suggested that estrogen is an important factor in preserving lifetime under starvation by increasing β-oxidation and ketogenesis (Jikumaru et al., 2007). In a study by Kovács and colleagues, male and female rats were treated with an exogenous ketone MCTG-supplement over a 17-month period. They observed that the female rats had a significantly higher BHB blood concentration in comparison to the male rats between the age of 2 – 11 months but dropped after 12 months and were eventually surpassed by male rats in terms of BHB blood concentration (Kovács et al., 2020). Interestingly, mice enters menopause around 12 months of age and shows significantly lower estradiol levels at 12 months compared to 6 month old female mice (Bellofiore et al., 2021). Based on these studies, it seems that estrogens are involved in the metabolism of ketone bodies. Our results correspond well with this theory.

The additional increase in BHB for KDF at the last day may be explained by the complete removal of exogenous substrates for gluconeogenesis. When exogenous substrates for gluconeogenesis is removed, as in fasting, the last option becomes the endogenous substrates and is completely dependent on adipose tissue lipolysis for glycerol, protein catabolism for glucogenic amino acids, and lactate. In a study done by Wang and colleagues, carbon isotope gluconeogenic substrates was used in fasting mice to investigate the preferential substrates for gluconeogenesis. The researchers concluded that glycerol was the dominant overall contributor to glucose production from gluconeogenesis in mice following short-term and prolonged fasting (Wang et al., 2020). If glycerol is a preferred substrate for gluconeogenesis during fasting, it is reasonable to believe that adipose tissue lipolysis is accelerated during fasting, not only to support energy homeostasis from fatty acids and ketones, but also to maintain glucose homeostasis. Adipose tissue lipolysis results in liberation of glycerol and free fatty acids from stored triglycerides (Chouchani & Kajimura, 2019). The increased production of free fatty acids may further enter ketogenesis to produce more ketone bodies, which may explain the additional increase in BHB observed in KDF mice.

Baseline blood glucose for CD was surprisingly significant different from KD/KDF in both genders. It is worth mentioning that non-fasting blood glucose levels depend on a variety of factors such as stress, post-prandial period, and exercise (King et al., 2020; McCowen et al., 2001; Nagy & Einwallner, 2018). As of the baseline measurements, the leg of the mice was shaven just minutes before the measurements were performed. The shaving process and the fact that the technique notably was a little bit “rusty” in the beginning, may have stressed some of the mice exceedingly, possibly creating a stress response that may have elevated the blood glucose levels. Apart from of the blood glucose CD baseline phenomenon, the graphs almost behave in a mirrored manner in comparison to BHB levels through the experiment. This complies well with the theory of ketogenesis increasing as glucose availability decreases, working together to maintain energy homeostasis in an antagonistic manner.

An important contributor in maintaining metabolic homeostasis in both genders are estrogen (Mauvais-Jarvis, 2011). Estrogen is the predominant female sex hormone (Chai et al., 2014). In a study by Yan et al., glucose homeostasis in female ovariectomized mice were investigated (Yan et al., 2019). By using subcutaneous administration of estradiol, the researchers noticed a suppressive effect on gluconeogenesis by lowering fasting blood glucose levels, in the ovariectomized female mice, but also estradiol treated male mice. The difference

in development and stabilization of glucose levels as observed between the genders in our results may be explained by different estrogen levels in the mice.

5.4 Inflammation is affected by ketogenic diet and not fasting

Rather unexpectedly our results showed that the ketogenic diet reduced acute inflammation induced by LPS whereas introducing fasting in addition to the ketogenic diet did not. This was shown both by *in vivo* and *ex vivo* imaging of NF-κB as well as by cytokine measurement. Specifically, TNF-α and IL-6 were reduced whereas IFN-γ, IL-10 and IL-1β was not affected by KD. We initially hypothesized that BHB was responsible for a potential anti-inflammatory effect, but KDF (which had the overall highest blood BHB concentration) did not seem to give the same effect as KD. Additionally, female mice fed KD both had higher BHB levels than corresponding male mice but displayed no reduction in inflammation. Thus, the ketogenic diet apparently caused a diminished inflammatory response to LPS compared to high-fat fed control mice independently of BHB.

We are not the first to see that KD can reduce inflammation in mice. In a study by Nandivada et al., inflammatory markers were investigated after feeding mice a KD or carbohydrate dominant, low-fat control diet for 4-weeks (Nandivada et al., 2016). NF-κB transcription levels in the liver were measured after LPS-induced inflammation, with similar LPS-doses used in our experiment (1mg/kg). What Nandivada and colleagues found, was a clear reduction in hepatic NF-κB activity in the KD mice compared to the low-fat control mice, in both the postprandial state and overnight fasting. This complies well with our results observed for the KD group, but not for our KDF group. Interestingly, in the Nandivada study, they used 12E% fat in their control diet, whereas we used 75E% fat, which is a very high-fat diet. and colleagues suggested that the anti-inflammatory effect of the diet may have been related to the elevated levels of BHB, but that the exact mechanism was unclear to them.

Circulating cytokines can be used as biomarkers to assess inflammation. IL-6 and TNF-α are both potent cytokines involved in NF-κB activation (De Simone et al., 2015; Ilchovska & Barrow, 2021). The trend observed in KD showing a reduction in TNF-α and IL-6 compared to CD and specifically KDF is somewhat confusing at first. Mu and colleagues found a reduction in both TNF-α and IL-6 in KD fed mice compared to standard fed mice after administration of bleomycin (Mu et al., 2021). Notably, the inflammatory stimuli were different from our experiment, and their experiment lasted longer with a duration of 7 weeks.

However, in the study mentioned earlier by Nandivada et al., cytokines were also measured. They found, on the other hand, an increase in both TNF-α and IL-6 levels in the postprandial state for the KD mice, but a reduction in both TNF-α and IL-6 after fasting for 14 hours (Nandivada et al., 2016). Nandivada and colleagues suggested that insulin resistance caused by an elevation of circulating free fatty acids in their KD mice may have been a key explanation to this observation. They further suggested that when mice undergo prolonged fasting, insulin resistance normalize, and release of cytokines may be less prominent. This contradicts our results observed in KD and KDF.

A possible explanation from the elevated inflammatory markers in KDF, may be due to the mobilization of adipose tissue and increased lipolysis. It is known that cytokines regulate the metabolism of adipose tissue (Asterholm et al., 2014; Martins et al., 2018; Menezes-Garcia et al., 2014). Lacerda and colleages investigated inflammatory markers in lean, chow-fed, 24-hour fasting mice and observed an increase in IL-6, TNF-α and IL-10 in the adipose tissue of the chow-fed, 24-hour fasting animals (Lacerda et al., 2019). However, they did not find the same increase in mobilization of the cytokines in obese, 24-hour fasting mice as in lean mice. If lean, fasting mice has a higher concentration of inflammatory cytokines in adipose tissue, it may be possible that the increased lipolysis in the cytokine rich adipose medium allows for cytokine release into the bloodstream. As stated in Coppack's review on cytokines and adipose tissue, IL-6, but not TNF-α appears to be released systemically by adipose tissue (Coppack, 2001), which could explain the elevated IL-6 levels in KDF observed from our results.

IL-6 has been shown to be secreted from contracting skeletal muscle, at least in humans (Pedersen & Febbraio, 2008). As observed from our results, lean mass was higher in male mice compared to females, and this may also explain the elevated IL-6 response that was observed for the males. Moreover, mice fasting for just 6 hours has been shown to increase plasma IL-6 concentration (Wueest et al., 2014), which may explain the increase in IL-6 at least for male KDF mice.

However, Miyauchi et al., found suppressed TNF-α and IL-6 levels in a 12-hour fasting, mouse model for liver injury (Miyauchi et al., 2019). This was notably not with LPS-induced inflammation, but with liver injury provoked through ischemia and reperfusion. In the same study, Miyauchi and colleagues also found suppressed NF-κB activity and NLRP3 expression in the liver tissue of fasting mice. There seems to be conflicting evidence whether fasting

protects against inflammation, and the potential anti-inflammatory effects may be context dependent.

If BHB was responsible for any anti-inflammatory effects, it would be expected that females which maintained higher BHB levels would benefit more from the KD and KDF than males. However, this was not the case as observed in NF-κB activity or IL-6. This suggests further that BHB itself is not responsible for the reduction in inflammation for KD.

A third explanation, which is more speculative is based on the condition of fasted mice, which may have influenced the outcome. When we dissected the mice, we noticed that most KDF mice had material in their intestines despite fasting for about 13 hours prior to dissection. We figured that the KDF mice must have eaten some of the wooden chips in their cage and this may have impacted the outcome. No scientific data that supports the theory of a potential increase in inflammation in mouse after eating wooden material was found. It would be interesting to re-do the experiment with removing any edibles during the fasting period for KDF mice.

We speculate that ketosis per se is responsible for the anti-inflammatory effect since we see this clear difference between high fat and ketogenic diet, but it is challenging to argue that this is due to ketone bodies. As Nandivada suggested in their study, the mechanism in which ketogenic diets express anti-inflammatory effects is unknown. Apart from BHB being a possible mediator, they further suggested that KD may decrease oxidative stress, primarily through upregulation of glutathione peroxidase (Nandivada et al., 2016). Notably, several studies on KD and oxidative stress do indeed demonstrate several oxidative modulatory effects of the diet (Shimazu et al., 2013; Sullivan et al., 2004; Veech, 2004; Ziegler et al., 2003). This is a different and comprehensive field of research on KD, but it could be interesting to investigate the oxidative modulatory effects of the KD, and how it translates to inflammation.

5.5 NF-κB activity evaluated by in vivo imaging and ex vivo imaging

Inflammation is a driver for many chronic diseases. A central component involved in inflammatory regulations are the transcription factor NF-κB. The NF-κB-luc mouse model allowed us to assess grade of inflammation by measuring NF-κB activity with the usage of IVIS Lumina II.

The *in vivo* analysis revealed that NF-κB activity is significantly increased from 2 hours to 5 hours past LPS injection. Carlsen and his colleagues used a similar *in vivo* imaging technique on NF-κB-luc mice and found an increase in NF-κB activity from 2,5 hours to 4 hours past LPS administration (Carlsen et al., 2002). Moreover, IκB has been shown to be maximally reduced 45 minutes after LPS administration (Hobbs et al., 2018), while IKK is activated minutes after LPS administration, but reaches peak activation after approximately 45 minutes, at least in mouse cell cultures (Covert et al., 2005). Covert et al. also found that LPS causes a sustained NF-κB nuclear translocation for at least 3 hours.

Even though the 5-hour NF-κB analysis have been on the "decreasing" side of the peak activity, we still managed to find a significant time effect which corresponds well with the results of the previous studies mentioned. The whole-body images show particularly strong luminescence from the abdominal region corresponding to the liver. The liver is an essential organ in maintaining inflammatory homeostasis, through the control blood flow, capillary permeability, migration of leukocytes into tissues and secretion of inflammatory cytokines. Moreover, hepatocytes and Kupffer cells express PRR which binds PAMPs or DAMPs. Upon binding, DAMPs and PAMPs are phagocytosed and eventually degraded (Robinson et al., 2016). The liver is responsible for the removal and detoxification of certain pathogenic molecules, such as LPS (Jirillo et al., 2002). To verify if the in vivo images corresponded to the liver activity, liver, intestine, and brain was taken out and assessed for luminescence ex vivo. *Ex vivo* liver radiance correlated with *in vivo* radiance likely because of the elevated inflammatory responses generally seen in liver. Since the ROI for the *in vivo* analysis covered both thoracic and abdominal area, it is likely that a greater *in vivo – ex vivo* NF-κB activity correlation would be observed if the *in vivo* ROI were restricted to only the liver area.

We found a significant higher NF-κB activity in liver amongst male mice. This gender effect may be explained by the female hormone estrogen, which has been shown to possess some anti-inflammatory effects on NF-κB, but has also been demonstrated to have suppressive effects on IL-6 and TNF-α levels (Straub, 2007). In mouse cells, the administration of the estrogen hormone, estradiol, after an LPS induced inflammatory response has indeed been demonstrated to block DNA-binding and transcriptional activity of RelA subunit by preventing nuclear translocation (Ghisletti et al., 2005). Moreover, in a mice study by Cheng et al., female ovariectomized mice were first sensitized to ovalbumin to induce an airway inflammation (Cheng et al., 2019). The mice were further treated with estradiol, and the expression of NLRP3 components were assessed. After estradiol administration, protein- and

mRNA expression of NLRP3, caspase-1 and IL-1β were suppressed, suggesting that estradiol suppresses NLRP3i assembly by downregulating the expression of NLRP3 components. Our results here correspond well with other studies demonstrating a lesser inflammatory response in female mice.

5.6 Methodological considerations

5.6.1 Experimental design

One issue in the experimental design that made the statistical analyses somewhat problematic, was the low *n* per group when genders were separated. A consideration that would have made the statistical analyses easier and more accurate, was to only use one gender. This would enable more observations per group, allowing any possible significant group differences to be detected more accurately. On the other hand, if we did use only one gender, we would not have been able to detect some interesting findings between the genders. As we initially did not intend to investigate differences between genders, the restriction to one gender may have benefited the strength of the analytical outcome of the inflammatory markers.

5.6.2 The experimental diets and energy intake

Creating an extremely high-fat diet that would remain firm and not and sieve through the feed tray, due to the warm and humid climate inside the animal lab was quite challenging. Several methods to optimize the KD in a way where it could be placed in a feed tray to monitor feed intake were assessed. We initially wanted to use more unsaturated fats or MCTG fats, but this would make the feed more liquid in room temperature. Due to this, we were forced to use predominantly saturated fats, mainly from dairy butter and cocoa butter, which is high in the long-chain fatty acids C16:0 and C18:0 (Caligiani et al., 2016). We also added quite a lot of cellulose to bind up the tiny amount of water that was present due to the dairy butter and lard. The diets, in particular the KD, may not have been optimal in terms of the sources of fats, but we had to prioritize a firm, non-dripping feed, on behalf of the unsaturated fat content. Even though we eventually did manage to create a KD with the sensory qualities we desired, it still may have caused some inaccuracy regarding the energy intake, as fatty particles were scattered inside the cage (on paper bedding, running wheel and the animals themselves), and were difficult to include in the feed intake measurements. Let alone, the abundance of saturated fats in the diets were unrepresentable for a typical ketogenic diet and may have

impacted the results from the inflammatory markers. By using a different vessel for the feed inside the cages, we may have enabled a more accurate estimation of energy intake, while also enabling the incorporation of other desired sources of fats.

5.6.3 Characterization of weight and body composition

We hypothesize that the duration of experimental period was not sufficient to observe any changes in the weight of the animals. Implementing a longer experimental period may have been a solution to this issue.

5.6.4 Development of blood BHB and non-fasting blood glucose

Weak planning and delayed deliveries of the keto strips prevented us from performing all the BHB and blood glucose measurements we intended to do. This would of course be fixed by securing all the required tools in advance to the experiment. Non-fasting blood glucose is generally not a good way to measure glucose homeostasis, due to the lack of control for the postprandial state of the animals. This means that the blood glucose levels may vary greatly depending on the time and how much the mice ate before the measurements, which would particularly impact the CD group with its significant amount of carbohydrates. However, measuring fasting blood glucose for the animals would make the experiment more comprehensive. Regardless, measuring non-fasting blood glucose levels is still beneficial to measure the effects of the diets on postprandial blood glucose levels. In an experiment where glucose homeostasis and insulin are emphasized, it may be more favorable to measure fasting blood glucose.

5.6.5 Analysis of NF-κB activity

One issue regarding the timing of the *in vivo* analysis was that the blood sampling, dissection, and organ harvest was more time consuming than expected. This forced our intended 4 hour past LPS-injection analysis to be extended to 5 hours past LPS-injection. If we preempted a few more minutes per mice to the blood sampling, dissection, and organ harvest, we may have been able to complete the procedure of the analysis according to the planned 4 hour past LPS-injection. For the *ex vivo* analysis, our ambition was to analyze other organs as well, such as the brain, for NF-κB activity. As harvesting the brain was yet more time consuming, we had to prioritize our main organs of interest and so we focused on the liver and intestines. Analyzing other organs may also have been possible with some better time preemptions.

5.6.6 Cytokine profiling and quantification

Some issues with the analysis of the cytokines were encountered, in which several cytokines were not detected. This was mainly due to a bead-number that was too low or did not fit the reference range of the standard curve. When the bead-number of a particular cytokine was too low to be detected (< OOR), the datapoints were excluded completely from the analysis. However, this may have been a mistake. Excluding data from cytokines that were too low to be detected may have given a false result by removing the data from individuals with the lowest levels of certain cytokines. What could have been an idea, was to still include any < OOR values and for instance adjusted and defined these data as the minimum detectable value. Besides, the whole issue with undetectable beads may very well have been avoided if the 96-well plate was successfully analyzed the first time. Since the plate was washed and more detection buffer was added before the second attempt, the samples were diluted significantly. This may have affected the detection of some beads.

6 Conclusion

Based on our results, the mice remained similar weight and lean mass but increased in bodyfat following both the KD and CD. KD fed females maintained higher blood BHB levels compared to males, while KD fed males maintained higher non-fasting blood glucose levels. Fasting increases BHB levels more than adherence to a KD. We suspect that BHB itself does not promote any anti-inflammatory effects after LPS-induced inflammation. The KD may provide minor anti-inflammatory effects through suppression of TNF-α, IL-6 and NF-κB activity after LPS-induced inflammation.

More research, with some changes in experimental design would be useful in assessing the potential anti-inflammatory effect of the KD further.

7 Future perspectives

Additional analyses could give a more accurate and clearer picture of the orchestra of effects from the KD. Such analyses could be more consecutive measurements of BHB to assess at which day the exact peaking occurred, as well as measurements of fasting blood glucose and insulin levels. Biomarker analysis for cardiovascular health, such as cholesterol, triglycerides and serum amyloid A could be of interest. Liver triglyceride analysis could be interesting to evaluate the state of non-alcoholic fatty liver disease. Feces sampling and analysis could be

useful in assessing the influence of the KD on microbiota. Assessment of estrogen levels could be beneficial in determining different gender effects of the KD. It would also be interesting to implement a standard chow-fed control group to compare the effects from KD with a standard diet.

With the current literature on KD and chronic diseases, it could be interesting to create a disease model in mice for i.e., liver- or colon cancer and KD feeding. This could give a better idea of the implementation of KD for certain chronic diseases. It is worth mentioning that our experiment was of a moderate duration, and the effects observed may only have been beneficial short-term. A longer experimental period, i.e., 8-weeks or more could give a better picture of the safety of the KD long-term.

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Appendices

Appendix A – Materials

A. CHEMICALS AND REAGENTS

Chemical/reagent product name	Cat. No.	Supplier
DL-β-hydroxybutyric acid sodium salt	H6501	Sigma-Aldrich, St. Louis, MI
Ethylenediaminetetraacetic acid (EDTA)	03690	Sigma-Aldrich, St. Louis, MI
Lipopolysaccharide (LPS)	L2880	Sigma-Aldrich, St. Louis, MI
IsoFlo® Vet 100% Isoflurane	002125	Zoetis, New Jersey, USA
D-Luciferin Firefly	L-8200	Biosynth, Basel, Switzerland
Rompun vet		Bayer Animal Health, Germany
Fentadon vet		Dechra Veterinary Products AS, Oslo, Norway
Zoletil Forte		Virbac, Sollihøgda, Norway

Appendix B - Kits

Kit product name	Cat. No.	Supplier
D-3-hydroxybutyric acid assay kit	K-HDBA	MegaZyme (Neogen), Bray, Ireland
IL-1 beta Mouse ProcartaPlex Simplex Kit	EPX01A-26002-901	Invitrogen (ThermoFischer) Inc, MA, USA
IL-6 Mouse ProcartaPlex Simplex Kit	EPX01A-20603-901	
IL-10 Mouse ProcartaPlex Simplex Kit	EPX01A-20614-901	
IL-18 Mouse ProcartaPlex Simplex Kit	EPX01A-20618-901	
IFN gamma Mouse ProcartaPlex Simplex Kit	EPX01A-20606-901	
TNF alpha Mouse ProcartaPlex Simplex Kit	EPX01A-20607-901	
ProcartaPlex Mouse Basic Kit	EPX010-20440-901	

Appendix C – Instruments and Software

Product	Software	Supplier
Accu-Check glucometer		Roche Diagnostics, Mannheim, Germany

Freestyle precision Neo		Abbott, Fornebu, Norway
IVIS Lumina II	Living Image®	PerkinElmer, Walthamn, MA, USA
	R Studio (version 2021.09.02)	
TD-NMR Minispec LF50		Bruker, Billerica, MA, USA

Appendix D – Websites

Bioplex-200 System *Instruction manual*	http://rai.unam.mx/manuales/umyp_Manual_Bioplex.pdf
MegaZyme ***Assay protocol***	https://www.megazyme.com/documents/Assay_Protocol/K-HDBA_DATA.pdf

Appendix E – Raw data

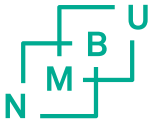
Liver radiance

Group	Gender	Individual	Avg radiance	Organ
Control	Male	1	1,53E+05	Liver
Keto/fasting	Male	2	8,05E+04	Liver
Keto/fasting	Male	3	1,49E+05	Liver
Keto/fasting	Female	4	4,53E+04	Liver
Keto/fasting	Female	5	4,70E+04	Liver
Control	Female	6	6,60E+04	Liver
Control	Female	7	6,39E+04	Liver
Keto	Female	8	4,44E+04	Liver
Keto	Female	9	3,33E+04	Liver
Keto	Female	10	5,23E+04	Liver
Control	Male	11	2,42E+05	Liver
Control	Male	12	7,97E+04	Liver
Keto	Female	13	4,98E+04	Liver
Keto	Male	14	7,06E+04	Liver
Keto	Male	15	2,60E+04	Liver
Control	Male	16	3,77E+05	Liver
Keto/fasting	Female	17	5,91E+04	Liver
Keto/fasting	Male	18	1,08E+05	Liver
Keto/fasting	Male	19	2,65E+05	Liver
Control	Female	20	8,16E+04	Liver
Control	Female	21	6,31E+04	Liver
Keto/fasting	Female	22	3,51E+04	Liver
Keto/fasting	Female	23	5,13E+04	Liver
Control	Female	24	5,81E+04	Liver
Keto	Female	25	7,88E+04	Liver

Keto	Female	26	1,02E+05	Liver
Control	Female	27	9,94E+04	Liver
Keto	Male	28	9,88E+04	Liver
Keto	Male	29	8,44E+04	Liver

Cytokine profiling

			IFN gamma	**TNF alpha**	**IL 6**	**IL 1 beta**	**IL 10**
Group	**Gender**	**Individual**	**Obs Conc**	**Obs Conc**	**Obs Conc**	**Obs Conc**	**Obs Conc**
Control	Male	1	81,11	41,85	49772,73	36,98	125,63
KDF	Male	2	-	74,57	33688,78	36,98	44,15
KDF	Male	3	43,42	-	52784,31	44,64	293,14
KDF	Female	4	35,31	174,99	15507,69	36,98	102,18
KDF	Female	5	47,39	-	25723,17	36,98	71,18
Control	Female	6	23,33	-	24119,32	16,21	128,5
Control	Female	7	286,86	66,38	35187,82	30,77	96,16
KD	Female	8	147,52	125,86	12441,87	27,46	238,26
KD	Female	9	20,38	74,57	8618,58	18,95	194,24
KD	Female	10	328,18	115,47	33844,02	66,72	139,84
Control	Male	11	224,55	145,4	19389,58	18,95	74,39
Control	Male	12	77,95	13,24	28270,47	13,24	164,74
KD	Female	13	75,1	16,21	9921,76	16,21	202,13
KD	Male	14	183,68	102,93	20556,71	9,92	137,02
KD	Male	15	693,1	91,19	29386,56	16,21	116,95
Control	Male	16	266,58	149,42	33175,99	9,92	148,23
KDF	Female	17	333,43	278,53	42782,42	62,72	217,75
KDF	Male	18	336,06	141,33	51831,33	5,96	212,57
KDF	Male	19	407,4	219,56	44397,55	17,6	202,13
Control	Female	20	128,56	82,21	18371,08	32,89	175,58
Control	Female	21	101,79	177,45	15885,54	40,88	196,87
KDF	Female	22	100,55	-	26328,09	14,76	108,14
KDF	Female	23	117,06	196,63	19061,1	40,88	331,93
Control	Female	24	46,07	217,31	12112,17	9,92	476,05
KD	Female	25	405,37	-	22994,87	13,24	222,91
KD	Female	26	86,46	12,91	27635,05	-	44,15
Control	Female	27	-	82,21	18856,14	32,89	12,3
KD	Male	28	282,34	52,63	24735,3	8,05	71,18
KD	Male	29	184,93	62,03	17025,01	-	-



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