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Effects of Dietary R, S-1, 3-Butanediol Diacetoacetate on Components of Energy Balance and Hepatic Health

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EFFECTS OF R,S-1,3-BUTANEDIOL DIACETOACETATE ON COMPONENTS ON ENERGY BALANCE AND HEPATIC HEALTH

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ABSTRACT

Objective: Exogenous ketone administration presents a possible treatment strategy for obesity and obesity-related diseases. The exogenous ketone ester, R,S-1,3-butanediol diacetoacetate (BD-$AcAc_2$) decreases adiposity and hepatic steatosis in high-fat diet-induced obese mice. In our previous work, carbohydrate energy was removed and replaced with the ketone ester to produce isocaloric diets. The current investigation aimed to determine whether BD-$AcAc_2$ decreases markers of hepatic steatosis and inflammation in lean mice on a high-fat, high-sugar (HFHS) diet without removing carbohydrate energy. **Methods:** Sixteen 11-week-old male C57BL/6J mice were randomized to one of two groups for 9 weeks (n = 8 per group): 1) Control (CON, HFHS diet) or 2) Ketone ester (KE, HFHS diet + BD-$AcAc_2$, 25% by kcals). **Results:** Body weight increased by 56% in CON (27.8 ± 2.5 to 43.4 ± 3.7 g, $p < 0.001$) and by 13% in KE (28.0 ± 0.8 to 31.7 ± 3.1 g, $p = 0.001$). The CON group had a greater accretion of total adiposity compared to the KE group ($p < 0.001$). Despite significant differences in body weight and adiposity, energy intake and expenditure were similar between groups. Nonalcoholic fatty liver disease activity scores for hepatic steatosis, inflammation, and ballooning were lower in the KE group compared to CON ($p < 0.001$ for all). Markers of hepatic inflammation [*Tnfα* ($p = 0.036$); *Mcp1* ($p < 0.001$)], macrophage content [(Cd68 ($p = 0.012$)], and collagen deposition and hepatic stellate cell activation [(*αSma* ($p = 0.004$); *Col1A1* ($p < 0.001$)] were significantly lower in the KE group compared to CON. **Conclusions:** BD-

$AcAc_2$ administration abolishes HFHS-induced markers of liver steatosis, inflammation, ballooning, and fibrosis in lean B6 mice. Future studies should explore the physiological and molecular mechanisms responsible for these findings.

KEYWORDS: fatty liver, energy balance, ketones, body fat

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LIST OF ABBREVIATIONS

3HB-BD	D-beta-hydroxybutyrate-(R)-1,3 butanediol
AcAc	acetoacetate
AcAc-CoA	acetoacetyl-CoA
ACC	acetyl CoA carboxylase
AEE	activity-induced energy expenditure
ALT	alanine transferase
ARG1	arginase 1
AST	aminotransferase
AT	adaptive thermogenesis
BAT	brown adipose tissue
BD-$AcAc_2$	R,S-1,3-butanediol diacetoacetate
BH-BD	bis-hexanoyl R-1,3-butanediol
BMR	basal metabolic rate
CCN1	cellular communication network factor 1
CD11b	cluster of differentiation 11b
CD163	cluster of differentiation 163
CD68	cluster of differentiation 68
COL1A1	collagen 1 alpha 1
CON	control group

CR	calorie restriction
DKA	diabetic ketoacidosis
DNL	*de novo* lipogenesis
ETC	electron transport chain
FASN	fatty acid synthase
FFA	free fatty acid
FM	fat mass
FT	facultative thermogenesis
G6pase	glucose 6-phosphatase
GGT	gamma-glutamyl transferase
HFD	high-fat diet
HFHS	high-fat, high-sugar
HMG-CoA	hydroxymethylglutaryl-CoA
HMGCL	hydroxymethylglutaryl-CoA lyase
HMGCS2	3-hydroxymethylglutaryl-CoA synthase
HOMA-IR	homeostasis model assessment for insulin resistance
HSC	hepatic stellate cell
IL-1β	interleukin-1 beta
KD	ketogenic diet
KE	ketone ester group
KS	ketone salts
LBM	lean body mass
LDL-C	low-density lipoprotein cholesterol

MCP1	monocyte chemoattractant protein-1
MCT	monocarboxylic acid transporter
MMP2	matrix metalloproteinase 2
MMP9	matrix metalloproteinase 9
MRC1	mannose receptor c-type 1
NAFLD	nonalcoholic fatty liver disease
NAS	NAFLD activity score
NASH	nonalcoholic steatohepatitis
Pgc1-α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PDGFβ	platelet-derived growth factor-beta
Pepck	phosphoenolpyruvate carboxykinase
QMR	quantitative magnetic resonance
QUICKI	quantitative insulin-sensitivity check index
REE	resting energy expenditure
RER	respiratory exchange ratio
RQ	respiratory quotient
SCOT	succinyl-CoA-3-oxoacid CoA transferase
TEE	total energy expenditure
TEF	thermic effect of food
Tfam	mitochondrial transcription factor a
TNFα	tumor necrosis factor alpha
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
VCO_2	volume of carbon dioxide output

VLDL-C	very low-density lipoprotein cholesterol
VO_2	volume of oxygen consumption
WAT	white adipose tissue
αSMA	alpha-smooth muscle actin
βHB	beta-hydroxybutyrate

CHAPTER 1: INTRODUCTION

Obesity and obesity-related metabolic conditions, such as nonalcoholic fatty liver disease (NAFLD), are major public health concerns. Over 40% of adults in the U.S. are currently living with obesity, with projected rates approaching 50% by 2030[1-3]. While its etiology is complex and multi-factorial, obesity results from a chronic positive energy balance[4]. Over the past 4-5 decades, there has been an increase in the consumption of energy-dense processed foods with high carbohydrate content, particularly added sugars[4,5]. Consumption of a high-fat, high-sugar (HFHS) diet is a major contributor to the incidence of obesity and obesity-related metabolic diseases[4,5], including NAFLD[6].

Macronutrients (i.e., carbohydrate, protein, and fat) are essential to life. Humans require a combination of these macronutrients in their diets for health and longevity. Research spanning decades has sought to determine the distribution of macronutrients required for optimal health. Historically, human populations have survived on diets with wildly differing proportions of carbohydrate, protein, and fat. Some populations consume more than 40% of their daily energy from fat, and others consume as little as 1% of their daily energy from fat[7]. It is commonly stated that "a calorie is a calorie," and reference is frequently made to the first law of thermodynamics. However, multiple bodies of evidence have shown that diets of varying macronutrient content produce different changes in body mass and composition. For example, research suggests that diets high in carbohydrate are an important determinant in the obesity epidemic[6,8].

With the industrialization of food supply came a surplus of food products high in carbohydrate and, consequently, an increase in the rates of obesity[4,5]. Alternate fuel sources are available as the body can metabolically shift into a state of ketosis by restricting carbohydrates through fasting or the ketogenic diet (KD). With decreased carbohydrate intake, insulin secretion can be stabilized at lower levels. Stored fat in adipose tissue will undergo lipolysis, generating free fatty acids (FFA) that can enter β-oxidation in the liver[5]. An excess of fatty acid-derived acetyl-CoA will be produced, eventually driving the body toward ketogenesis (described below)[5]. Ultimately, appropriately tailored diets with varying macronutrient compositions can help manage the obesity epidemic, with multiple diet regimens under investigation for the treatment of obesity and obesity-related metabolic diseases.

Calorie restriction (CR) is a well-established strategy to reduce body weight and adiposity. However, long-term success using this approach is often tempered by physiological responses to decreased energy intake, such as increased appetite, increased energy intake, and decreased energy expenditure. These responses blunt expected weight loss and promote recidivism and weight regain[9,10]. An important metabolic change that occurs during CR is an increase in circulating ketone body concentrations, which could provide a mechanistic explanation for the observed benefits of CR[11].

High-fat, low-carbohydrate KD produce weight loss through mechanisms that likely involve increases in circulating ketones and/or decreases in circulating insulin[12-14]. KD-mediated reductions in body weight may be attributed to reduced appetite and energy intake[15,16], maintenance of energy expenditure[15,17-19], lower concentrations of circulating insulin[14,15,20], and maintenance of skeletal muscle mass[14,15]. Studies in humans show that

very low-calorie, high-fat, and normocaloric KDs may also be treatment options for NAFLD, again demonstrating the potential beneficial effects of ketones independent of calorie consumption. While mechanisms have not been fully elucidated, improvements in markers of hepatic steatosis[20-24] and decreases in hepatic insulin[20,22], inflammation[22], and fibrogenesis have been observed[22]. However, poor compliance has led to varying degrees of long-term success with KD[13-15]. The robust and consistent beneficial effects of KD on components of energy balance and metabolism have spurred interest in pursuing nutritional ketosis exogenously.

The ketone ester, R,S-1,3-butanediol diacetoacetate (BD-$AcAc_2$), increases circulating ketone concentrations in rodents from 0.5 to 1.0 mM[25-28]. Studies from our laboratory show that BD-$AcAc_2$ decreases body weight and adiposity in obese mice on a high-fat diet (HFD)[25] and attenuates weight gain in lean mice on a low-fat diet (LFD)[19]. A follow-up investigation demonstrated that BD-$AcAc_2$ significantly reduced histological hepatic steatosis, inflammation, and NAFLD activity score (NAS) on a HFD[29]. In addition, BD-$AcAc_2$ decreased hepatic expression of profibrotic markers, increased markers of anti-inflammatory M2 macrophages, and reduced pro-inflammatory markers[29].

In previous studies, carbohydrate energy of the diet was reduced (25-30%) to accommodate the additional energy content of BD-$AcAc_2$ to create an isocaloric diet control[19,25,29]. Thus, our findings may be influenced by the decrease in carbohydrate energy. The purpose of the current investigation was to determine whether BD-$AcAc_2$ decreases the accretion of body weight and adiposity and markers of hepatic steatosis, inflammation, and collagen deposition in B6 mice on a HFHS diet. The findings of this

dissertation show that consuming the dietary ketone ester, BD-$AcAc_2$, recapitulates the physiological and metabolic phenotype previously observed under other nutritional approaches using a HFHS nutritional model. If these findings can be reproduced in humans, the clinical and practical implications are potentially important due to the limitation in currently available treatment strategies for hepatic steatosis and inflammation.

CHAPTER 2: LITERATURE REVIEW

Ketone Metabolism

Humans possess an elaborate physiological and metabolic response to overnutrition that promotes the diversion of excess energy to triglyceride production and storage in adipose tissue. The advantage of such a system lies in the capacity to efficiently store energy for several weeks to months during periods of prolonged starvation. Within 24-48 hours of fasting or starvation, fatty acid-derived acetyl-CoA is diverted from the tricarboxylic acid (TCA) cycle, a metabolic pathway that breaks down nutrients to produce energy and high-energy molecules used in ATP production. Fatty acid-derived acetyl-CoA is then condensed with acetoacetyl-CoA (AcAc-CoA) to produce the ketones, acetoacetate (AcAc), acetone, and beta-hydroxybutyrate (βHB)[30,31]. Ketones are secreted from hepatocytes in a 4(βHB):1(AcAc) ratio with total ketone concentrations ranging from 1-2 mM after 24 hours and up to 6-7 mM following 1-2 weeks of starvation[31-34]. Once in circulation, βHB and AcAc are transported into extrahepatic tissues by monocarboxylate 1 transporters (MCT1)[31]. βHB is reduced to AcAc and is eventually oxidized in extrahepatic mitochondria to acetyl-CoA, where it enters the TCA cycle and eventually enters oxidative phosphorylation for ATP production.

Ketogenesis

Ketogenesis is a "spillover" pathway that takes place primarily in the mitochondria of the liver to produce ketone bodies from β-oxidation-derived acetyl-CoA[35]. There are several physiological and biochemical regulators, such as glucagon and insulin, TCA cycle activity, and lipolysis of fatty acids from triglycerides[35,36]. During periods of fasting or starvation, glycogen is depleted from muscle and liver, and insulin and glucagon induce fatty acid mobilization from adipose tissue for fatty acid β-oxidation[31,36]. Any available oxaloacetate and glucogenic amino acids will be used for gluconeogenesis to maintain glucose levels, which slows TCA cycle activity and causes an excess of β-oxidation-derived acetyl-CoA[35]. Once acetyl-CoA availability exceeds citrate synthase activity or the availability of oxaloacetate to form citrate, it will enter ketogenesis, which will lessen the demand for glucose by extrahepatic tissues and limit protein degradation[35].

Two acetyl-CoA molecules condense in the first ketogenic reaction to form AcAc-CoA via thiolase (Fig. 1)[35]. AcAc-CoA and a third acetyl-CoA are condensed to hydroxymethylglutaryl-CoA (HMG-CoA) by 3-hydroxymethylglutaryl-CoA synthase (HMGCS2)[31,35,36]. This rate-limiting step is upregulated by glucagon and downregulated by insulin[31,36]. Hydroxymethylglutaryl-CoA lyase (HMGCL) cleaves HMG-CoA to produce acetyl-CoA and AcAc[31,35,36]. AcAc is the primary ketone body and common precursor to both acetone and βHB. AcAc is reduced to βHB by βHB dehydrogenase in a reversible reaction[31,35,36]. The redox potential of this reaction is controlled by βHB dehydrogenase and is largely reliant on the ratio of NADH to NAD+. AcAc may also undergo spontaneous degradation to acetone, which is excreted as a waste product in the

breath and urine[31,35,36]. The reduction of AcAc to βHB is favored because βHB is less likely to spontaneously degrade to acetone, making it a more stable molecule for transport and substrate metabolism in extrahepatic tissues[31,35,36]. Finally, AcAc and βHB are transported into extrahepatic tissues via monocarboxylic acid transporters (MCT1 and MCT2) for terminal oxidation[31,35,36].

Ketolysis

Ketone bodies produced in the liver are 1) excreted in the urine and breath, 2) diverted to lipogenesis or cholesterol synthesis, or 3) catabolized in the mitochondria of extrahepatic tissues to acetyl-CoA, which can be used in the TCA cycle (Fig. 2)[35]. In extrahepatic tissues, βHB is oxidized to AcAc via βHB dehydrogenase. Succinyl-CoA donates its CoA in a rate-limiting step to convert AcAc to AcAc-CoA via succinyl-CoA-3-oxoacid CoA transferase (SCOT)[35,36]. This reaction bypasses the irreversible reaction catalyzed by HMGCS2. Thiolase then catalyzes a reaction to free CoA, producing two molecules of acetyl-CoA[35,36]. Ketolysis occurs in all tissues except for the liver and red blood cells. The liver lacks SCOT and red blood cells do not have mitochondria, so they lack the machinery needed to utilize ketone bodies[37].

The transport and metabolism of βHB is preferred over AcAc because the oxidation of βHB produces more ATP, and the stability of βHB as a substrate reduces the risk of spontaneous degradation of AcAc to acetone during transport out of hepatic mitochondria and into extrahepatic tissues. In the oxidation reaction of βHB to AcAc, NADH is also produced, which can be used as a substrate in the electron transport chain

(ETC) to produce additional ATP. The use of NADH in the ETC continues to drive the oxidation of βHB to AcAc, creating more acetyl-CoA for the TCA cycle.

Signaling and Nutrient Roles

In addition to their role in metabolism, ketones have important signaling and nutrient roles that are poorly understood. Numerous studies demonstrate endogenous ligand activity for βHB for the hydroxycarboxylic acid receptor 2 (HCAR2). Activation of HCAR2 by βHB regulates adipose lipolysis, a response that is thought to reduce substrate flux into the liver as part of a negative feedback loop to limit circulating concentrations of ketones[31]. This event is fundamentally different from diabetic ketoacidosis (DKA), where the absence of insulin leads to a state of unsuppressed lipolysis and excessive ketone production to concentrations at or above 20 mM[28,30,35]. The receptor is relatively ubiquitous, and activation with βHB has been shown to reduce inflammation in macrophages and other tissues. Therefore, βHB activation of HCAR2 could have potential roles in treating a number of chronic diseases[31].

GPR43, a short-chain fatty acid receptor (SCFA), is activated by AcAc under ketogenic conditions[38]. As previously stated, plasma ketone concentrations increase during fasting, but SCFA concentrations have been shown to decrease[38,39]. This suggests that AcAc becomes the primary ligand for GPR43 in plasma under ketogenic conditions[38,39]. GPR43 is highly expressed in adipose tissues and is involved in the modulation of inflammation and metabolism through gut microbiota[38,39]. Stimulation of GPR43 by AcAc promotes lipid utilization in plasma by plasma lipoprotein lipase activation[38]. Overall, AcAc plays a key role in maintaining body weight and lipid levels

through GPR43-mediated lipid metabolism, which could contribute to the development of therapeutic treatment strategies for a number of metabolic disorders.

Histone deacetylases (HDACs) are a family of proteins that suppress gene expression by deacetylating lysine residues on histone and non-histone proteins[38]. They are involved in major biological functions, such as DNA repair, stress response, cell cycle, and transcription[40]. Butyrate was the first HDAC inhibitor identified. Butyrate's structural similarity to βHB led to interest in the ketone body's activity as an HDAC inhibitor. βHB has since been identified as an endogenous and specific inhibitor of class I HDACs. Increasing βHB circulation via fasting or exogenous administration increases global histone acetylation[41]. βHB-mediated HDAC inhibition is correlated with changes in transcription, including genes encoding oxidative stress resistance factors[31,41]. Thus, treatment with βHB results in protection against oxidative stress[31,41].

NLR family pyrin domain containing 3 (NLRP3) inflammasome is a multiprotein intracellular complex activated by diverse damage-associated signals such as reactive oxygen species, endoplasmic reticulum stress, excess $Ca^{2+,}$ toxins, excess glucose, and more[39,42]. Its activation contributes to a proinflammatory and proatherogenic environment[39], which means its ablation attenuates a number of chronic diseases, including type 2 diabetes, atherosclerosis, bone loss, and Alzheimer's disease[42]. Identifying mechanisms that mediate NLRP3 inflammasome activation may provide insights into the development of chronic disease. βHB administration has been shown to exert anti-inflammatory properties by inhibiting NLRP3 inflammasome[39,43]. It is suggested that βHB acts as a signaling molecule by binding to HCAR2 or serving as an HDAC inhibitor[42]. However, βHB inhibits NLRP3 inflammasome activity independently

of binding to HCAR2, thus avoiding competition for receptor occupancy[42]. Therefore, ketone administration represents a therapeutic strategy to reduce systemic inflammation[39,43]. Ultimately, the response to individual ketone bodies varies based on target cell type, ketogenic rate, and physiological variations. While many questions remain, increasing evidence suggests both metabolic and signaling roles for ketones that have important effects on regulating metabolism during starvation and re-feeding.

Ketogenic Diet

Originally developed in the 1920s as a treatment for epilepsy, low-carbohydrate, high-fat diets, i.e., the KD for the control of epileptic seizures due to the production of ketones[44-46]. The observed benefits were similar to those produced while fasting, which was also considered a treatment for epilepsy, partially due to the rise in ketones levels observed within 48 hours[47]. Historically, the KD consisted of a 4:1 ratio of fat-to-carbohydrate and protein[48]. Current guidelines for the KD are high fat (65-80%), moderate protein (15-30%), and low carbohydrate (<5%)[44]. As anti-epileptic drugs came along, the KD lost popularity until the 1990s, when it became a recognized treatment for intractable epilepsies[44,45]. Interest in ketone metabolism and the KD grew as it became a therapeutic approach for various disease states.

Energy Balance

While the KD has become a well-established treatment for epilepsy, in recent years, it has also been used to treat cancer, metabolic disorders, and neurodegenerative diseases and weight loss[44-46]. The KD is similar to a Western diet in that it represents a

high-fat, high-energy state; however, it is also unusual as it simultaneously activates pathways associated with a fasted state. For example, mice fed a KD had increased expression of genes associated with fatty acid oxidation and suppressed fatty acid synthesis enzymes[14]. In rodent models, KD feedings show decreases in body weight and adiposity and alterations in energy expenditure compared to standard chow and high-fat controls[14]. Mice fed a KD lost weight without differences in energy intake compared to mice on a standard diet or HFD[14]. Also, KD-fed mice experienced similar changes in body weight compared to mice on a calorie-restricted diet despite consuming more calories[14]. These results suggest that KD-fed mice have increased metabolic rates. Trials in both rodents and humans have shown increases in total energy expenditure (TEE) with KD feedings compared to control[12-14]. The increase in energy expenditure may be caused by increases in uncoupling proteins associated with oxidative phosphorylation and mitochondrial function[14,15]. However, other investigations have shown decreased or no differences in energy expenditure, but the methods used to measure energy expenditure vary and include metabolic chambers, indirect calorimetry, and doubly labeled water[12-14,17]. It has been argued that decreases in body weight are not due to increased energy expenditure, but may be related to decreased energy intake from the KD's anorexigenic effects, energy loss in the form of ketones via urine or sweat, or methodological flaws[15,17,18]. One important metabolic adaptation associated with the KD is that the decrease in basal metabolic rate (BMR) that usually accompanies weight loss does not appear to occur[19]. Ultimately, the mechanisms controlling energy balance while on a KD are poorly understood and warrant further investigation.

Obesity

Adherence to the KD leads to significant improvements in multiple anthropometric and biochemical parameters and has proven to be quite beneficial for individuals with obesity and with or without diabetes mellitus. There is ample evidence supporting the KD as an approach to decrease body weight and adiposity; some benefits are reported independent of changes in body weight[48-51]. Improved glycemic control and insulin sensitivity are often reported after initiation of the KD, with some studies showing improvements significant enough to negate the need for insulin and/or oral glycemic medications in Type 1 and 2 diabetic populations[49,50,52]. However, the KD may be associated with an increased risk of hypoglycemic episodes, so diet initiation must be done with medical supervision[49]. Improvements in blood-lipid profiles, including increases in high-density lipoprotein-cholesterol (HDL-C) and decreases in low-density lipoprotein cholesterol (LDL-C), triglycerides, and total cholesterol, are frequently observed[49,50]. Conversely, some reports indicate the KD may result in unfavorable alterations in blood-lipid profiles, such as increased LDL-C, triglycerides, and total cholesterol[51,53]. These alterations could be due to factors such as increased saturated fat intake or poor adherence to the reduced dietary carbohydrate protocol[51]. Therefore, the KD could potentially put those susceptible to cardiovascular disease at increased risk. Clinical trials have shown that participants report reduced appetite and hunger while following the KD[54]. Multiple mechanisms of action, both direct and indirect, may be responsible for this phenomenon. Alterations in metabolic hormones, such as ghrelin and leptin, fcould impact appetite and hunger[12,54]. It has been suggested that increased satiety associated with increased protein intake could also be a factor; however, high-protein KD

have been shown to decrease appetite more than a high-protein diet alone[54]. Overall, there are multiple pathways in which the KD may produce favorable alterations in anthropometric and biochemical profiles, many of which remain unclear.

Nonalcoholic Fatty Liver Disease

NAFLD is a spectrum of liver diseases that results from fat deposition in the liver[55]. NAFLD affects individuals with metabolic syndrome, which involves at least three of the following disease states: obesity, hypertension, diabetes mellitus, hypertriglyceridemia, and hyperlipidemia. While some mechanisms are unclear, insulin resistance, oxidative stress, inflammation, and fibrosis play an important role in the pathogenesis of NAFLD[55]. The progression of NAFLD is generally reversible if the underlying cause is identified and treated before it reaches fibrosis. Because it has been shown that KD effectively reduces insulin resistance, oxidative stress, lipogenesis, and inflammation, it has been explored as a possible treatment for NAFLD. Improvements in steatosis have been reported with KD adherence, with one investigation showing complete regression of fatty liver[22-24,56]. Additional studies have documented decreases in liver volume, with and without improvements in steatosis[56-58]. Liver enzymes, aminotransferase (AST), alanine transferase (ALT), and gamma-glutamyl transferase (GGT), can be evaluated as a measure of liver function, with increases typically indicating liver damage. Improvements in AST and ALT levels have been noted after KD initiation[23,59]. However, these results have been variable as some investigations, particularly those involving very low-calorie KDs, have reported no changes in liver enzymes when compared to levels before initiation of the diet[60-62]. This may be due to

study design, as research involving very low-calorie diets is typically short-term, with outcomes measured only at baseline and the end of the study, sometimes making it challenging to interpret results. When following a very low-calorie diet, improvements in liver pathology may result from the weight loss itself. However, improvements in liver pathology seem to be more dependent on macronutrient content, specifically ketosis, when in caloric excess[55]. Clinical trials have also shown decreases in inflammation, *de novo* lipogenesis (DNL) genes, and hepatic triglyceride content with KD adherence[22,24,59,63,64]. Overall, there is mounting evidence that the KD is an effective treatment strategy against NAFLD, but the role ketosis plays may go beyond carbohydrate restriction and weight loss. More research is needed to determine the mechanisms involved and the possible benefits of ketosis independent of dietary composition.

Over the years, it has become apparent that long-term adherence to KDs is difficult[22,44,48]. Difficulty in complying is due to many things, including palatability, lack of knowledge, and support[65]. Additionally, there are concerns about the safety of a long-term, HFD. It is well known that high fat intakes are associated with increased cardiovascular disease risk, and some KD studies showed increases in LDL-C and very low-density lipoprotein cholesterol (VLDL-C). High-fat diets are also contraindicated for several disease states, such as pancreatitis and liver failure[48,66]. Individuals with diabetes mellitus are of particular concern due to the increased risk of hypoglycemic episodes being a possibility[48,49]. While NAFLD can present in lean individuals[67], ultimately NAFLD is the liver's response to excess body fat. Therefore, there are concerns about the safety of a long-term, HFD because of the potential to lead to NAFLD. The answer to

these concerns warrants further investigation, but one solution may be the supplementation of exogenous ketones. Many of the benefits of KD are due to the presence of ketone bodies and their involvement in physiological processes. With dietary ketone supplements, it may be possible to increase circulating ketones and reap the benefits of a ketogenic state without the challenges of a restrictive diet.

Exogenous Ketones

As a ketone body, βHB yields more free energy and produces fewer reactive oxygen species than glucose and fatty acids[68]. Compared to pyruvate, it has a higher heat of combustion and yields 30% more energy[69]. βHB also regulates gene expression and diminishes inflammation by directly blocking inflammasomes[68,69]. Furthermore, βHB is an important signaling metabolite and reduces oxidative stress by increasing histone acetylation through activation of HDACs[68]. While the benefits of βHB are plentiful, the varying success of dietary interventions to increase βHB and the downsides of the KD itself led to an interest in developing a way to produce sustained ketosis without the KD. Many of the diseases in question are caused by some form of metabolic dysfunction, and βHB, a naturally occurring ketone body, may provide an alternative in the form of an ingestible ketone[68,70]. Ingestion of an exogenous ketone can quickly raise blood ketone concentrations, similarly to the KD, without requiring strict dietary control[68,69].

By using dietary ketone supplements, it is possible to accurately manipulate blood βHB and AcAc concentrations in a way that is not possible with interventions reliant on endogenous ketone production. One method of increasing ketone concentrations is via ingestion of ketone salts (KS). However, reports show that a large amount of salts are

required to achieve low levels of ketosis (~1 mM blood βHB concentrations) and along with the burden of the high salt load make these forms of ketones less practical for many individuals[70]. Consumption of a ketone ester can reach blood βHB concentrations similar to what occurs after several days of fasting but within only minutes or hours of ingestion[68,71]. Investigations in rodents have shown increases in blood ketones >5 mM after ketone ester ingestion[70,71]. Clinical trials used body weight-adjusted doses and reached blood βHB concentrations of 3-5 mM in approximately 1 hour[69,70]. Ketosis is often maintained for up to 6 hours, with the peak at about 30-60 minutes[69,70,72]. Exogenous ketone ingestion makes it possible to achieve ketosis even after consuming a meal. Peak βHB concentrations were significantly decreased by approximately 1 mM after the high-carbohydrate meal but still ranged from 1.3 to 3.5 mM[70]. Once exogenous ketosis has been achieved, it can be maintained for several hours with continuous nasogastric infusions or serial ketone ester drinks[70]. With the increases in blood ketone concentrations, decreases in FFA, glucose, and triglycerides have also been reported in both fed and fasted states[70,71]. The reduction in both glucose and FFA is unique to exogenous ketosis and makes it possible to simultaneously produce a benefit and decrease a potential risk of the KD[70]. Overall, dietary ketone ester consumption repeatedly increases blood βHB concentrations, providing evidence that exogenous ketones in the form of an ingestible ketone ester can induce nutritional ketosis without requiring adherence to the KD.

Ketone Monoesters

Ketone esters are now commercially available in two primary forms. Ketone precursors, such as butanediol, are esterified to βHB or AcAc to form a ketone monoester or diester[73,74]. Artificially inducing ketosis via ingestion of a dietary ketone monoester is one method of elevating blood ketone levels. Compounds such as R-3-hydroxybutyrate-R-1,3-butanediol were developed to evaluate the pharmacokinetics of an orally administered ketone monoester[69]. The ketone monoester is hydrolyzed to R-βHB and R-1,3-butanediol by esterases found in the gastrointestinal tract, blood, and liver[65,68,69]. R-1,3-butanediol is converted to D-βHB and AcAc in the liver by alcohol and aldehyde dehydrogenases (Fig. 3A)[68,69]. The ketone bodies are then readily metabolized in multiple tissues through the normal process of ketolysis. The molecular structure of ketone esters seems preferable because it is possible to mimic endogenous ketosis by providing the substrates needed for hepatic ketogenesis rather than delivering ketones directly into circulation[75].

Ketone monoesters have been delivered orally and via a nasogastric (NG) tube with similar results in humans and rodents[69-71]. Ketone monoesters have been shown to increase βHB concentrations about 50% higher than other exogenous ketones, such as KS[70]. The difference in maximum βHB concentrations is likely due to ketone monoesters containing 99% physiological R-βHB enantiomer[68,70]. Additionally, the time it took to reach peak concentrations (T_{max}) was about half as long as the T_{max} for KS, suggesting that R-βHB has a faster rate of metabolism[70]. Breath acetone concentrations are also higher after consuming a ketone monoester compared to KS, indicating that R-βHB is more readily converted to acetone than S-βHB[70]. The metabolic fate of R-βHB tends to

lean towards the production of acetyl-CoA for energy metabolism, but the metabolism of S-βHB is unclear[69,71].

Consumption of dietary ketone monoesters does not always come without side effects. In rodents, transient hypoglycemia has been documented[71]. This could pose a risk in disease states such as diabetes mellitus. In some human trials, severe adverse events such as vomiting, chest pain, abdominal pain and distention, diarrhea, and constipation have been reported after high doses of the ketone monoester[69,73]. Some trials have also documented mild to moderate headaches, dizziness, and lethargy[69,73]. In these cases, all adverse events were resolved by the end of the study[69,71,73]. Many of these symptoms, such as nausea and bloating, have mostly been reported immediately after consuming the ketones and are likely caused by the rapid intake of a large dose of unpalatable drinks[73]. Developing a more pleasant-tasting drink will be necessary to evaluate the side effects of a dietary ketone monoester accurately.

Ketone Diesters

BD-$AcAc_2$ is a ketone diester developed to induce therapeutic ketosis in animal and human models[76,77]. BD-$AcAc_2$ was originally used as parenteral nutrition in pigs and dogs but has since become a possible therapeutic strategy for seizures, neurocognitive diseases, and metabolic diseases[76,77]. Like ketone monoesters, BD-$AcAc_2$ is hydrolyzed to butanediol and AcAc by esterases in plasma and tissues[77]. However, once in the liver, R,S-1,3-butanediol is oxidized to both the R- and S-isoforms of βHB (Fig. 3B)[77]. R-βHB is oxidized to AcAc and eventually to acetyl CoA in extrahepatic tissues. Conversly, S-βHB forms S-β-hydroxybutyl-CoA, which is one of the final intermediates in the β-

oxidation of fatty acids[25]. Bis-hexanoyl R-1,3-butanediol (BH-BD) is another ketone diester under recent investigation. It is quickly metabolized to produce ketogenic precursors such as hexanoic acid and R-1,3-butanediol[74,75]. R-1,3-butanediol is oxidized to R-βHB, and hexanoic acid undergoes β-oxidation and subsequent ketogenesis[74]. Both BD-$AcAc_2$ and BH-BD have been used to achieve and sustain ketosis effectively[75-77].

BD-$AcAc_2$ diester compound has a neutral pH, so the damage to peripheral tissues is minimized when given parenterally[77]. This provides another possible avenue for ketone administration in addition to oral and NG tube administration, both of which have been done successfully in animals and humans[74-77]. By using BD-$AcAc_2$, it is possible to produce a rapid and continued increase in all three ketone bodies, including both R- and S-βHB, without concomitant sodium overload observed with KS ingestion[76-78]. Compared to R-βHB, the non-physiological S-βHB seems to have a lower rate of metabolism as it remained elevated for more extended periods following administration[70]. In rodents, S-βHB produced R-βHB and AcAc but generated fewer reducing equivalents than ketogenesis from long-chain fatty acids[25,79]. Further, S-βHB metabolism does not increase the free NADH:free NAD+ ratio, suggesting that oxidation of endogenous fatty acids is not inhibited[25,79]. Others[80] demonstrated lower oxidation of S-βHB compared to R-βHB and superior substrate utilization for fatty acid and sterol synthesis. Stubbs and colleagues[70] used racemic mixtures of R- and S-βHB and found that S-βHB is oxidized to a lesser extent and is not a major oxidative fuel in humans. However, the fate of R-βHB in rodent trials was oxidation in peripheral tissues[25]. Approximately 25% of R-βHB is metabolized to S-βHB, so it is possible that the differences observed could be due to lesser use as an energy substrate and greater use in fatty acid oxidation, as seen with S-

βHB alone[25]. While the H+ load from acute administration did not lead to metabolic acidosis in rodents, it is possible that the lower rate of metabolism could lead to the accumulation of ketone bodies in circulation in humans, especially after multiple ingestions of the ketone[70,76]. The kinetics of the novel ketone diester BD-BH are not fully understood. Still, it also may deliver more AcAc than a ketone monoester since it provides ketogenic precursors and relies on endogenous ketone production, which would generate physiologic ratios of the ketone bodies[74].

While ketone diesters are as unpalatable as the monoesters, rodents showed increased tolerance when the diester was slowly integrated into their diet over 7 days[81]. In humans, side effects similar to those reported after ketone monoester ingestion have been observed, including mild to severe vomiting, dizziness, nausea, and reflux[75,78]. However, these side effects are typically infrequent and transient[75]. While the bitter taste of the ketone could contribute to feelings of nausea, tolerability increases when the serving size is gradually increased[75].

Ketone Salts

The second class of exogenous ketones contains varying amounts of both R-βHB and S-βHB bound to a cation, such as sodium, potassium, or calcium, creating a ketone salt[70,73]. The pharmacokinetics of KS are poorly understood due to their racemic nature, as they contain both the bioactive R-βHB and S-βHB, the latter of which is not normally found in the blood[68,70,82]. The onset of ketosis appears to be delayed due to the presence of both isoforms of βHB and their differenes in metabolism[70,82]. Compared to a ketone monoester, breath acetone concentrations and peak R-βHB concentrations were lower,

but S-βHB concentrations remained elevated for 8 hours following KS ingestion[70]. These results suggest that S-βHB may be less readily oxidized and processed in different pathways or cellular compartments than R-βHB[70].

Ketosis has been achieved via intravenous infusion and oral ingestion of KS[83]. However, it has been shown that reaching low levels of ketosis requires a high amount of βHB salts[70]. This excessive mineral intake can increase the risk of severe gastrointestinal side effects and potentially fatal conditions such as salt-induced hypertension[73,82]. Increases in urinary and blood pH have also been documented and could be responsible for side effects such as dizziness, nausea, and vomiting[70]. Additionally, high salt intakes could cause alterations in fluid homeostasis and blood pressure, leading to water retention in the gut, headache, or nausea[73]. While KS supplements have been shown to produce ketosis, the results are variable and often come with more frequent and severe adverse events[73,82].

Energy Balance

Energy balance is the state in which energy intake equals energy expenditure, resulting in weight maintenance[84,85]. TEE is comprised of multiple components: resting energy expenditure (REE; the energy needed to support basic metabolic needs while an individual lies quietly awake), activity-induced energy expenditure (AEE; the energy cost of physical activity and exercise), the thermic effect of food (TEF; the energy spent to process ingested food), and the energy necessary for thermoregulation[86]. REE is approximately 3-10% higher than the BMR, the energy necessary for vital bodily functions[86-89]. Both REE and BMR are largely determined by body size and composition

and are positively correlated with body weight and fat-free mass[86]. AEE is the most variable component of TEE and depends on the individual's lifestyle[86,90,91]. While food intake affects multiple components of TEE, it primarily affects TEF, with varying effects based on the macronutrient composition of the meal and the daily variation of the individual[86,92,93].

Energy balance is a very complex process that is crucial for survival. Multiple unique mechanisms counteract energy imbalances, including adaptive thermogenesis (AT) and facultative thermogenesis (FT)[86]. AT and FT, which are influenced by SNS activity, leptin, and other hormones, involve the production of heat in response to environmental factors to protect from cold exposure and to regulate energy balance after dietary changes[86]. Brown adipose tissue (BAT) is a major site of AT, where thermogenesis occurs with the uncoupling of mitochondrial substrate oxidation from ATP production and heat from fatty acid oxidation[86,94,95]. Cold exposure and certain food ingredients activate BAT and contribute to TEF[86,96,97]. These signals also induce UCP1 in white adipose tissue (WAT), a phenomenon known as 'browning'[86,98].

Methods for Assessing Energy Balance

Many investigations, including the present one, may be interested in the impact of nutritional interventions on energy expenditure. The ability to accurately measure TEE, or its components, largely depends on the available instrumentation. Several methods are used to estimate energy expenditure, including doubly labeled water, direct calorimetry, prediction equations, and indirect calorimetry. Doubly labeled water requires the use of stable isotopes and mass spectrometry to measure energy expenditure[99,100]. Direct

calorimetry is a closed system that directly measures heat production to obtain a measurement of energy expenditure[99]. While both of these methods are highly accurate, they are also costly in terms of time and labor, so they are typically reserved for research purposes. Additionally, using these methods makes it difficult or impossible to measure components of energy expenditure, such as expenditure during activity phases. Prediction equations vary in accuracy depending on the variables required for calculation and the population for which the prediction equation is being used. For example, the Mifflin-St. Jeor equation may be more appropriate for normal-weight populations while the Owen equation may be better suited for individuals with obesity[101]. Finally, indirect calorimetry uses the volume of oxygen consumed (VO2) compared to the volume of carbon dioxide produced (VCO2) to estimate energy expenditure with Weir's equation[99]. The ratio of VCO2/VO2 is called the respiratory exchange ratio (RER), which can be used as a measure of substrate utilization[99,102]. At the tissue level, substrate utilization is called the respiratory quotient (RQ), with RQ values being 1.0 for carbohydrate, 0.8 for protein, and 0.7 for fat and alcohol[102,103].

Indirect calorimetry is conducted with single or multichannel systems. In single channel systems, a single chamber is connected to a single analyzer[102]. Animals are measured one at a time, with the measurements taken the entire time the animal is in the chamber. Single channel systems are ideal for measuring specific components of energy metabolism (i.e., BMR, cost of physical activity). However, unless multiple systems are available, which is costly, measuring multiple animals is a slow process, as the usual output is about 5 animals per week[102]. With multiple channel systems, several chambers can be ventilated at the same time. Typically, systems include 8, 16, or 32 chambers, but

they still only use one analyzer and cannot measure more than one chamber at a time[102]. Because of this, the animals are measured for short periods throughout the measurement period with a purging cycle between each measurement to rid the system of leftover gases from previous animals[102]. Depending on the measuring and purging cycle chosen, animals can be measured for shorter, more frequent samples spread evenly throughout the day. This will require more purging time, so animals will be measured about 4% of the time they are in the chamber[102]. Alternatively, taking longer, less frequent samples will reduce the time needed for purging, so animals will be measured about 10% of the time they are in the chamber[102].

As previously mentioned, single channel systems are designed to measure energy metabolism components, such as BMR and the cost of physical activity. They are also good for measuring acute effects of drugs or compounds that may impact energy metabolism[102,104,105]. However, single channel systems are sometimes too small to contain food or water, so they are not ideal for measuring daily energy needs since animals will be unable to live in the chamber for any extended period[102]. Multiple channel systems chambers are usually larger, with space for food, water, nesting, and movement, which allows for the measurement of daily energy needs[102]. While they are not useful in measuring specific components of energy metabolism, multiple channel systems can provide details about daily energy expenditure, which is ideal for the present investigation.

Energy Intake and Composition

The actual energy content of foods may differ from the calculated and absorbed energy due to differences in digestibility and structure[86,106,107]. For example, including energy dense foods, such as nuts, that have a high lipid content in a diet does not necessarily negatively affect body weight and may even support weight loss[86,107]. This may be due to increased appetite control, increased TEF, or discrepancies in the available metabolizable energy[86,107]. The Atwater Factor is used to calculate metabolizable energy on the basis of 4 kcal/g protein, 4 kcal/g carbohydrate, and 9 kcal/g fat, but recent evidence shows this may not be accurate for certain food types, such as nuts[86,108,109]. Based on measurements in feces and urine, the metabolizable energy was 16-20% less than the Atwater calculations[86,108,109]. This could be due to food structure, which can limit the accessibility of digestive enzymes, and reduce the intake of energy[86]. While the effect of structure on actual energy content has been mainly demonstrated by nuts, a similar effect may be extended to other energy sources.

Energy intake increases energy expenditure through the TEF, which represents about 10-15% of energy expenditure[86]. With indirect calorimetry, TEF can be measured through the assessment of VO2 consumption and VCO2 production surrounding a meal. However, this assumes that all metabolic processes consume O2 and produce CO2, which is not always true[86,110]. Insulin resistance and abdominal adiposity can reduce the thermic effect of a meal, thereby reducing TEF and overall energy expenditure[86,111]. Insulin regulates substrate flow and utilization by increasing glucose oxidation and inhibiting lipid oxidation, but this is impaired in the presence of abnormal insulin sensitivity[86,112]. Additionally, as the caloric content of the meal increases, TEF increases

proportionally[86,93,113]. However, it is important to consider the macronutrient content of the meal as this affects postprandial energy expenditure as well. Generally, TEF exhibits a greater increase with a high protein meal than with meals higher in carbohydrate or lipids[86,114]. Furthermore, the macronutrient sources should be considered as well because they are metabolized differently, which may explain the extent and speed of TEF increase. For example, medium-chain triglycerides increase TEF more than long-chain triglycerides[115,116], unsaturated fats more than saturated fats[117], and fiber-rich carbohydrates increase TEF more than other carbohydrate sources[86,111].

Effects of Exogenous Ketones

Consumption of exogenous ketones may produce beneficial alterations in energy balance and, subsequently, overall health. Studies in rodents have shown decreases in body weight and adiposity with two of the original ketone esters available[19,25,118]. This could be due to decreased energy intake, altered energy expenditure, increased energy loss, or another mechanism that has yet to be identified[19,25,84]. Studies have shown decreases in energy intake with both BD-$AcAc_2$ and ketone monoester (D-β-hydroxybutyrate-(R)-1,3 butanediol; 3HB-BD) ingestion and no changes in energy intake with BD-$AcAc_2$ ingestion[19,25,118]. Additionally, the effects of a ketone-supplemented diet on energy expenditure have been equivocal, and in many rodent trials, the primary outcomes have not focused on energy balance. Some investigations in rodents show increases in REE and TEE on a diet supplemented with BD-$AcAc_2$ and 3HB-BD[25,118]. Conversely, another study in rodents examining the concentration-dependent effects of BD-$AcAc_2$ found no change or decreased energy expenditure at various ketone levels[19,84].

Ketone energy loss is another possible explanation for the energy imbalance, but energy losses through breath, urine, and feces are challenging to measure in rodents. An investigation in rodents measured feces output while on a low-fat diet and found that ketone-fed mice had 50% greater total fecal loss[84]. With bomb calorimetry, it was found that fecal energy loss could explain only 25% of the difference in adiposity between groups[84]. Finally, a human trial found that the amount of R-βHB excreted in the urine represented approximately 1.5% of the total amount consumed, suggesting the metabolic fate is oxidation in peripheral tissues[70]. Little is known about the metabolism of S-βHB. In rodents and humans, the S enantiomer appears to have a lower rate of metabolism than R-βHB and is not a major oxidative fuel[25,70,80]. In contrast, it has been shown that it may be used to a greater extent for fatty acid oxidation rather than as an energy substrate[25]. The differences in the metabolism of R- and S-βHB could contribute to the differences observed in energy expenditure and loss. Future studies should aim to determine their metabolic fates and contributions to energy balance.

Obesity

Obesity is a complex disorder characterized by excess body weight and fat accumulation, which alters anatomy and physiology, resulting in harmful metabolic, biomechanical, and psychosocial consequences[119]. Individuals with obesity have an increased risk of developing obesity-related metabolic abnormalities, such as NAFLD, type 2 diabetes, cardiovascular disease, cancer, and reduced life expectancy[120,121]. Although the etiology of obesity has not been fully elucidated, it may involve genetic predisposition, environmental and lifestyle factors, socioeconomic status, and dietary

behaviors[4,121]. Ultimately, obesity results when energy intake exceeds energy expenditure, resulting in prolonged positive energy balance and excess energy storage[4,122]. The availability of energy-dense, nutrient-replete foods has made it easy to overconsume and gain excess body weight.

Metabolic and Physiological Effects

Individuals with obesity often exhibit other metabolic and physiological effects secondary to excess body weight and adiposity. Obesity is often accompanied by an increase in macrophages and other immune cells in adipose tissue, partially due to tissue remodeling in response to adipocyte apoptosis. These immune cells secrete proinflammatory cytokines[123,124]. Excess secretion of proinflammatory adipokines leads to low-grade systemic inflammation in some individuals with obesity[124,125]. Hydrolysis of triglycerides releases FFA, which are transported to sites where they can be used in metabolic pathways, often resulting in elevated plasma FFA[124]. Lipids are also found in liposomes, small cytoplasmic organelles close to mitochondria in many cell types with excess fat. Liposomes in hepatocytes can increase in size, forming large vacuoles and subsequent pathological states, including NAFLD, steatohepatitis, and cirrhosis[124,126]. Elevated FFA, inflammatory adipokines, and lipid intermediates in non-adipose tissue contribute to impaired insulin signaling and insulin resistance[124,125,127].

Regulation of Obesity

The brain, specifically the hypothalamus, relies on information from the body and environment to regulate food intake. The hypothalamus receives and interprets satiation

signals (i.e., Agouti Related Peptide, Neuropeptide Y) to alter energy intake[122,128]. Peripheral organs and organ systems, such as the liver, adipose tissue, and gut, are also involved in energy balance regulation. Neural and hormonal messages from these organs and organ systems inform the brain about the body's energy status[122]. Peripheral signals, including leptin, insulin, and gut peptides, provide information on energy stores and food consumption. Leptin and insulin signaling primarily reflect long-term nutritional status while gut peptides (ghrelin, PYY, GLP-1, and CCK) provide information on current, meal-to-meal nutritional status[16]. These signals affect energy intake, energy expenditure, and nutrient metabolism as they influence feelings of satiety and hunger[16,122]. Therefore, dysfunction in generating or interpreting these messages is a factor in obesity.

Prevention and Treatment

Dietary interventions for the prevention and treatment of obesity and NAFLD have been tried previously with modest success. Even modest weight loss (>5%) can improve obesity- and NAFLD-related outcomes, but many of the approaches are not sustainable long term due to poor adherence[6,120,124]. CR is a well-established approach to reduce body weight, adiposity, and disease[129]. While CR has proven to be an effective weight loss strategy, physiological adaptations occur over time that blunt weight loss as the body's evolutionary defense mechanisms resist any further reductions in weight[9]. Specifically, reductions in energy expenditure and fat-free mass appear to moderate the level and duration of CR-induced weight loss[9]. Additionally, the sustainability of CR may be limited by increased feelings of hunger and decreased satisfaction of appetite, especially in an obesogenic environment conducive to overfeeding[4,10]. These factors and

the difficulty in maintaining CR long-term contribute to backsliding, and subsequent weight regain[9]. Pharmaceutical treatment options are also available when lifestyle modifications fail to manage obesity. These anti-obesity drugs target the following mechanisms: 1) decreasing fat absorption, 2) decreasing appetite and caloric intake, and 3) increasing energy expenditure[119,130]. While the use of pharmaceuticals has proven to achieve weight loss, many side effects and contraindications influence adherence and lead to withdrawal[119].

Bariatric surgery is the final option for the treatment of obesity. While this is reserved for more severe cases of obesity, surgical procedures have escalated in the last few decades[124]. While bariatric surgery may be more effective than lifestyle or pharmaceutical interventions, they are associated with greater risks[124,131-133]. The three primary types of bariatric surgery include gastric banding, Roux-en-Y gastric bypass, and vertical-sleeve gastrectomy[124]. On average, patients regain up to 10% of their lowest weight after 10 years, with more frequent complete weight regain after the gastric banding procedure[124]. This has led to concerns about safety, efficacy, and high reoperation rates, which has reduced the use of gastric banding[124]. Other limitations of bariatric surgery include cost, risk of short- and long-term complications, and weight regain in up to 20% of patients[124,132]. Overall, it is unclear if any available pharmaceutical or surgical treatment options are sustainable or effective enough to replace dietary treatment strategies.

Effects of Exogenous Ketones

Exogenous ketones provide an opportunity to examine the effects of ketone bodies on appetite or metabolism without strict dietary restrictions. Ketone esters, the primary exogenous ketone in recent research, can be used to induce and sustain ketosis in humans and animals. Appetite suppression, possibly due to alterations in signaling hormones and gut peptides, is a potential mechanism by which weight loss is achieved and maintained in a ketotic state. In a rodent trial, KME was added to chow over a 6-day period and there was a decrease in ad libitum food intake and a rapid decrease in body weight, which later stabilized[118]. The KME-fed mice had a 2-fold increase in leptin concentrations compared to control mice[118], which may explain the reduction in food intake. BD-$AcAc_2$ administration consistently reduces weight gain and increases weight loss in rodents[19,25,84]. It appears that there is a dose-dependent response to BD-$AcAc_2$ as mice consuming higher doses (30% of total energy) weighed 24% less than control mice while mice consuming 25% of their total energy from the ketone weighed 14% less than controls[19]. However, there were no differences in energy intake between mice on the 25% BD-$AcAc_2$ diet and control mice, providing further evidence that the effects of exogenous ketones on appetite regulation may be concentration-dependent[19]. The weight-responses to BD-$AcAc_2$ are consistent in mice in thermoneutral conditions as well[84].

In human studies, KME ingestion reduced postprandial ghrelin, GLP-1, and PYY compared to glucose ingestion[69,134,135]. A more recent investigation in humans demonstrated that consuming a KME drink had no effect on leptin concentrations, but did suppress ghrelin production 2 h after baseline[136]. Additionally, KME ingestion resulted in less desire to eat and more satisfaction compared to glucose ingestion[136]. Other trials in

humans demonstrated that KME-consumption did not alter ghrelin or leptin concentrations or the perception of hunger[137,138]. Because these findings are inconsistent, caution should be taken when suggesting that exogenous ketones result in improved appetite control. Much of the existing research involves use of KME, which has a different metabolic fate than BD-$AcAc_2$, so it will be important for future research to investigate BD-$AcAc_2$'s effects on appetite specifically.

Nonalcoholic Fatty Liver Disease

Liver conditions of various severity of injury and fibrosis fall under the umbrella term NAFLD. There has been a relatively recent increase in NAFLD research due to global disease prevalence[139]. In the United States, NAFLD can be defined as 1) steatosis with $\geq$ 5% fat infiltration in imaging or histology and 2) no presence of alcohol, drug, or virally induced steatosis[140,141]. The development of NAFLD is generally associated with an unhealthy lifestyle, as there is mounting evidence that visceral adiposity and insulin resistance are common risk factors[140,142,143]. Thus, research design commonly uses dietary models, specifically high-fructose diets or HFD[140]. While research progress has been steady, a therapeutic agent has not been approved, so treatments typically consist of multimodal interventions, including weight loss, lifestyle modifications, and medication optimization[139,140].

Hepatic Metabolism

Liver biopsies are the gold standard for NAFLD diagnosis, and they help confirm diagnosis and assess the amount of hepatic damage[140]. The NAS is a validated grading

scale used to grade disease activity based on multiple components[140,144]. Each component has a minimum score (0) and maximum score (3) for steatosis, lobular inflammation, and hepatocellular ballooning[140,144]. Lower NAS typically indicates less severe disease progression, while higher scores indicate more severe disease progression [140,144]. Depending on the stage of disease, the presence or absence of fibrosis is also graded by scoring systems[145].

When defining pathogenic instigators of NAFLD, the liver's capacity to metabolize energy substrates (i.e., carbohydrate, fatty acids) is overwhelmed, leading to a toxic accumulation of lipids[139,146]. These metabolites can induce hepatocellular stress, injury, and apoptosis, resulting in fibrogenesis, genomic instability, and a predisposition for cirrhosis and hepatocellular carcinoma[139]. When fatty acids are provided in excess or their disposal is impaired, they may serve as substrates for lipotoxic species that induce ER stress and hepatocellular injury[139].

Identifying the sources and metabolic fates of fatty acids in hepatocytes is crucial for understanding the metabolic foundation of NAFLD. Fatty acids are primarily delivered to the liver from the blood following triglyceride lipolysis in adipose tissue, which is a process that is regulated by insulin[139]. Impaired signaling by insulin in adipose tissue, such as with insulin resistance, contributes to NAFLD through dysregulated lipolysis, leading to excessive delivery of fatty acids to the liver[139,147]. During inflammation, phosphorylation of kinases in adipocytes impairs insulin signaling[139]. Therefore, metabolic and inflammatory events in adipose tissue could drive the pathogenesis of NAFLD and present as potential therapeutic targets[139,148,149].

Another major source of fatty acids is DNL, which is the synthesis of fatty acids from glucose and fructose. The increase in hepatic lipid content in patients with NAFLD is largely attributed to DNL[139,150]. While the use of glucose in DNL is highly regulated, almost all fructose is removed from portal vein blood by the liver, where it is then phosphorylated and used in DNL without regulation[139]. Rodent studies show that gut enterocytes are involved in fructose metabolism[151], and human studies show that the gut epithelium has limited absorptive capacity for fructose[152,153], so it is unclear how much fructose reaches hepatic portal circulation. After a large fructose load, phosphorylation of fructose in hepatocytes commits it to DNL, leading to depletion of ATP in the liver in humans and animals, which can augment cellular stress[139,154]. Consuming sugary drinks that contain sucrose or a mixture of glucose and fructose is associated with fat accumulation in the liver and, therefore, NAFLD progression[139,140,155-158].

Hepatic fatty acids are primarily metabolized by either β-oxidation or through esterification to form triglycerides[139]. Some individuals with NAFLD have mitochondrial dysfunction and impaired β-oxidation in the liver[139]. However, it is unclear if impaired mitochondrial function in the liver is the precursor to NAFLD or if mitochondrial dysfunction is a consequence of cellular stress related to the disease. The disposal of fatty acids through triglyceride formation is generally considered a protective response to excess fatty acids[159]. However, studies suggest that excess triglycerides may also contribute to metabolic abnormalities rather than just serving as a marker for those abnormalities[139,160,161]. When triglycerides are not exported from the liver to the blood as VLDL-C, lipid droplets are formed in hepatocytes, which is a defining feature of NAFLD[139].

Hepatic Protein Expression

A number of experimental models are used to elucidate the complex pathophysiological mechanisms of NAFLD. Furthermore, there are many causes of steatohepatitis pathophysiology, which complicates the establishment of a successful experimental model. Nutritional models based on overnutrition with adipose tissue enlargement and subsequent metabolic complications are useful for investigating mechanisms responsible for the development and progression of NAFLD.

Numerous proteins in the liver are involved in many different processes, including inflammation, fibrosis, lipid homeostasis, and glucose metabolism. Specifically, tumor necrosis factor alpha (TNFα) stimulates biological responses such as liver inflammation and regeneration, autoimmunity, and hepatocyte apoptosis and necroptosis[162]. Inflammatory cytokines such as TNFα are among the primary markers of NAFLD progression[163]. Additional hepatic inflammatory markers include cluster of differentiation 68 (CD68), expressed in macrophages, and cluster of differentiation 11b (CD11b), expressed in stellate macrophages[163]. Mice fed a high-fat, high-fructose, and high-cholesterol diet exhibited increases in these inflammatory markers[164,165].

Fibrogenesis and hepatic stellate cell (HSC) activation are also distinguishing traits of NAFLD progression, so it is important to identify markers related to these processes. Type 1 collagen, including collagen 1 alpha 1 (COL1A1), is synthesized by osteoblasts and fibroblasts in hard tissues like bone. Increased expression of COL1A1 in soft tissues, such as the liver or lungs, indicates fibrosis[166]. Platelet-derived growth factors, including PDGFβ, play a role in driving the proliferation of cells[167]. Thus, tissue fibrosis may reflect increased PDGFβ activity. Additionally, emerging evidence suggests

that inflammatory cytokines promote the up-regulation of PDGFs. Therefore, PDGF-mediated proliferation may be a hallmark of chronic inflammation, such as that seen in NAFLD[167]. Indeed, mice fed a high-fat, high-fructose, and high-cholesterol diet also presented with increased expression of these fibrogenic markers[164,165].

Prevention and Treatment

The treatment of NAFLD involves a multimodal intervention, including more conservative strategies such as lifestyle modifications and weight loss and more intense therapies such as surgery and medications. Regardless of weight status, patients are encouraged to participate in a healthy lifestyle approach, with treatment goals including lifestyle adjustments, increased physical activity, and alcohol/smoking cessation[6,140,168]. Mounting evidence demonstrates that significant weight loss benefits NAFLD outcomes, including improvements in biochemical tests, metabolic markers, liver histology, and quality of life[139,140]. Understandably, many diet regimens used for obesity treatment, such as CR and KD, are also used for treating NAFLD[169]. However, nutrient composition of these diets should be addressed as long-term adherence and patient-specific outcomes can be significantly affected by such restrictive diets[169].

More aggressive treatment strategies include surgery and medications. Investigations in humans found that there was resolution of NAFLD in the majority of patients who underwent Roux-en-Y gastric bypass or vertical-sleeve gastrectomy, with increased benefits seen in patients who also took diabetes medication, specifically pioglitazone[140,170,171]. However, postoperative outcomes should be considered because disease aggravation may occur in some patients after bariatric surgery[140,172]. Short- and

long-term morbidity and mortality rates post-bariatric surgery appear to be higher in patients with liver disease, complicating the discussions regarding the safety of bariatric surgery in this population[173].

Pharmaceutical research is advancing rapidly, but no specific pharmacotherapy strategies are currently approved[21,29,145,174]. Pathways associated with cell death, inflammation, metabolism, and the gut-liver axis are potential drug targets[145]. Because NAFLD is commonly associated with other co-morbidities, the safety and tolerability of any treatment are paramount due to possible drug-drug interactions[21]. Multiple preclinical and clinical trials are underway evaluating various compounds' efficacy, safety, and mechanism of action. Animal and human trials have had positive results, including improvements in fibrosis[175-177], inflammation[175,177], steatosis[178], insulin resistance[178], and NAS[175,176]. In several cases, investigators failed to reproduce the promising findings from preclinical studies[145]. The reason for this is multi-fold and includes factors such as the inability to reduce variability in human populations and differences in susceptibility to treatment[145]. Ultimately, no specific pharmaceutical treatment options are available or effective enough to replace dietary treatment strategies.

Effects of Exogenous Ketones

Dietary ketones present a potential therapeutic avenue to treat NAFLD. It is well known that ketone bodies are an alternate fuel substrate for the brain, and they exert anticatabolic effects in the liver, adipose tissue, skeletal muscle, and heart[29,34]. Further, hepatic macrophage metabolism of AcAc attenuates HFD-induced hepatic fibrogenesis[179]. Evidence also suggests that exogenous ketones have beneficial effects on

adiposity, thermogenic BAT, and glucose metabolism[19,25,84,118], which could benefit hepatic health outcomes. However, little research exists examining the effects of exogenous ketones on the liver, specifically NAFLD markers.

To date, only one investigation has demonstrated that dietary BD-$AcAc_2$ ameliorates HFD-induced hepatic steatosis, inflammation, and fibrosis in the liver[29]. Authors reported that BD-$AcAc_2$ feedings resulted in lower total NAS (steatosis, inflammation, and ballooning) compared to control mice[29]. Diets high in sugar, such as sucrose, increase hepatic DNL and, consequently, hepatic lipid content[139,150,180]. Therefore, reducing the carbohydrate content of the ketone diet may have contributed to the improvements observed by Moore and colleagues[29]. The ketone-fed animals also had lower total body weight, adiposity, and liver weights than control animals[29]. It is well-established that moderate weight loss can be beneficial for liver disease, but the ketone-fed mice exhibited greater improvements than pair-fed mice receiving similar energy provisions[29].

HSC activation is a primary driver of hepatic fibrogenesis, and it is induced by systemic metabolic dysregulation, signals from cellular injury, and proinflammatory immune cell activation[181,182]. AcAc has been shown to have a protective effect against diet-induced hepatic lobular fibrosis[179]. Indeed, a study in mice showed that dietary BD-$AcAc_2$ significantly reduced markers of HSC activation and transcriptional regulators of fibrogenesis[29].

Inflammation is a hallmark of NAFLD progression, and it is mediated by an imbalance in pro-inflammatory M1 macrophage activation and anti-inflammatory M2 macrophage reduction[183]. An investigation in rodents demonstrated that BD-$AcAc_2$

administration increased M2 macrophage marker cluster of differentiation 163 (CD163)[29], which plays an important role in anti-inflammatory and tissue regenerating processes. Additionally, BD-$AcAc_2$ feedings reduced the pro-inflammatory and proapoptotic signals of cellular communication network factor 1 (CCN1) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which may mitigate NAFLD progression[29].

The mitigated hepatic inflammation in the ketone-fed mice may be due to the anti-inflammatory properties of βHB, the primary ketone metabolite. There is evidence that βHB produces anti-inflammatory properties by inhibiting inflammasomes, particularly NLRP3[43]. In a mouse model of lipopolysaccharide-induced sepsis, ketone monoester administration reduced markers of NLRP3 activation and markers of hepatic inflammation, including interleukin-1 beta (*Il-1β*) and *Tnfα*[43]. Ultimately, the response to individual ketone bodies is important to consider as they may vary based on target cell type, ketogenic rate, and physiological variations[179]. For example, AcAc can promote oxidative stress and pro-inflammatory signaling, while βHB has been shown to reduce the transcription of IL-1β, an M1 pro-inflammatory marker[42,179]. However, when administered at higher concentrations, βHB may also have a pro-inflammatory effect and induce oxidative stress[184]. Thus, investigations should be attentive to the responses of distinct cell types and the total ketone body concentration and ratio provided when determining the role of AcAc vs. βHB in liver disease progression.

Limitations and Gaps in Knowledge

It was possible that a reduction in or prevention of weight gain with ketone supplementation would not occur in the context of a HFHS diet and without the removal of carbohydrate energy as previously observed on high-fat or low-fat diets[19,25,84]. If the current study demonstrated that a HFHS diet supplemented with BD-AcAc2 does not attenuate weight gain, these findings would have be equally valuable because they would likely provide helpful clues regarding mechanisms responsible for previous observations.

Additionally, changes in energy intake or expenditure were unable to explain alterations in body weight. Therefore, exploring additional mechanisms responsible for alterations in energy expenditure and body weight will be important for future investigations It could be that the animals are excreting more energy through urine, feces, or even breath. The methods for measuring energy could also be altered to provide a more complete picture of what is occurring during the experimental period. It is common for energy expenditure to only be measured once, which leaves gaps in understanding both the acute and chronic effects of ketone supplementation.

Finally, improvements in markers of NAFLD have been observed with ketone-supplemented HFDs, but increasing the sucrose content of the diet could have attenuated the effects of the ketone supplement and thereby limited the capacity of the ketone supplement to reduce markers of NAFLD. Ketone supplementation resulted in reduced body weight, adiposity, and NAFLD markers, so we must question if the benefits are due to weight loss, many of which have been well-documented, or the ketone. Overall, the findings of this dissertation will provide valuable insights into this possibility and will help to establish further hypotheses related to ketone supplementation.

CHAPTER 3: RESEARCH METHODS

Animals and Diets

Sixteen male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 9 weeks of age and single-housed with standard rodent chow for one week upon arrival to the UAB animal facilities. After seven days of acclimation, the mice were placed *ad libitum* on a HFHS diet containing 40% kcals from fat as lard, 40% kcals from carbohydrate as sucrose, and 20% kcals from protein as casein (Dyets Inc, #104925) for one week. At 11 weeks of age, mice were randomly assigned to one of two groups using a random number generator for an additional 9 weeks (n = 8 per group): 1) Control (CON; remain on HFHS; Dyets Inc, #104925) and 2) Ketone Ester (KE; HFHS + 25% ketone ester by kcals; Dyets Inc, #104926) (Table 1). The ketone supplement, BD-$AcAc_2$, was a gift from Disruptive Enterprises (Durham, NC).

Mice were single-housed in shoebox cages with filter tops, wood-chip bedding, and shredded paper nesting for the duration of the study. Cages, bedding, feeding containers, and water were changed monthly or as needed. Animals were maintained on a standard 12:12 light-dark cycle beginning at 0600h in temperature-controlled chambers at 22-23°C. All procedures and conditions were approved by the UAB Institutional Animal Care and Use Committee and conformed to the care procedures employed by the UAB Animal Resources Program.

Body Weight and Energy Intake

Body weight was measured daily in the UAB animal facilities. Animals were provided food daily and allowed to eat *ad libitum*. Diets were provided in feeding jars (Dyets, Inc, Bethlehem, PA), and the jars were refilled and weighed each day. The manufacturer performed bomb calorimetry to provide the energy content of the diets. Energy intake was calculated based on 4.537 kcal/g for the CON diet and 5.671 kcal/g for the KE diet.

Body Composition and Indirect Calorimetry

Body composition was measured using quantitative magnetic resonance (QMR; EchoMRI 3-in-1 version 2013; Echo Medical Systems, Houston, TX) immediately before randomization and following 9 weeks on CON or KE diets. Energy expenditure and locomotor activity were measured during the last week of the study using a TSE PhenoMaster indirect calorimetry system (TSE Instruments, Chesterfield, MO) in a temperature-controlled room at 22-23°C following protocols previously used in our lab[25]. In short, mice were acclimated to metabolic cages for 48h, with measurements obtained for ~24h the following day. TEE was determined by calculating the mean hourly energy expenditure over the measurement period and multiplying by 24. REE was measured by averaging the lowest observed metabolic rates over a pre-defined measurement period[102]. TEE and REE during the light and dark cycles (lights on at 0600) were determined by calculating the average hourly energy expenditure during the measurement periods. RER was determined by calculating the mean hourly RER over the measurement period and multiplying by 24. RER during the light and dark cycles (lights on at 0600) was

determined by calculating the average hourly RER during the measurement periods. Daily physical activity counts were determined by calculating the average hourly locomotor activity [horizontal (XT) and horizontal (YT)] over the measurement period and multiplying by 24.

Tissue Collection and Handling

Mice were fasted for two hours at 6:00 am and were euthanized by decapitation in a randomized sequence two hours later. Whole blood was collected from the trunk into 1.5 mL centrifuge tubes and immediately placed on ice for at least 30 minutes to allow for clotting prior to centrifugation at 1500 xg at 4°C for 10 minutes. Serum was isolated and stored at -80°C until analysis. Tissues were isolated, weighed, and snap-frozen in liquid nitrogen at -80°C until analysis.

Histology

An aliquot of snap-frozen liver was placed in 10% formalin, then embedded in paraffin, serially sliced, and stained with hematoxylin and eosin to examine liver morphology (histological steatosis, inflammation, and hepatocyte ballooning) by IDEXX RADIL (Columbia, MO). Images were obtained using an Olympus BX43 microscope (Waltham, MA) at 10X magnification. Liver specimens were graded and scored for differences in NAS (comprising fat accumulation, inflammation, and hepatocellular ballooning) by an individual blinded to the study groups for differences according to the Brunt Scale[185].

Biochemical Assays

Serum glucose and non-esterified fatty acid concentrations were determined using colorimetric assays (Cayman Chemical, Ann Arbor, MI and Sigma, St. Louis, MO, respectively). R-βHB concentration was measured from whole blood using a hand-held device (Keto-Mojo, Napa, CA). Serum insulin was measured using an ELISA kit from Alpco (Salem, NH). Quantitative insulin-sensitivity check index (QUICKI) and homeostasis model assessment for insulin resistance (HOMA-IR) were calculated as previously described[186,187].

Western Blot Analysis

Whole tissue liver homogenates were used for western blot analysis. Cell lysates were initially prepped in Triton X-100. Upon addition of Triton X-100, Laemmli gel loading buffer was added to the lysate (1 μg/uL) and boiled at 100°C for 10 minutes. Membranes were incubated for one hour in a blocking solution [5% dry milk in Tris-buffered saline (TBS), 0.1% Tween 20 buffer) after gel electrophoresis and transfer. Following blocking, membranes were incubated overnight in the primary antibody of interest. Membranes were washed three times for five minutes each with TBS-Tween 20 buffer and placed in a horseradish peroxidase-conjugated secondary antibody that was either anti-rabbit or anti-mouse specific (Nos. 7074 and 7076; Cell Signaling Technology, Danvers, MA). Blots (n = 8/group) were quantified using densiometric analysis (Image Lab 4.0). Differences in protein loading were controlled with amido-black staining. Corrections for differences in protein loading and transfer of all band intensities were quantified by laser densitometry using the total protein staining from

each lane. The primary antibodies used were: CD68 (No. 17832, Santa Cruz Biotechnology), CD163 (No. 58965, Santa Cruz Biotechnology), phosphoenolpyruvate carboxykinase (PEPCK; No. 271029, Santa Cruz Biotechnology), TRAIL (No.7877, Santa Cruz Biotechnology), and platelet-derived growth factor β (PDFGβ; No. 3169, Cell Signaling Technology).

mRNA Expression Analysis

A commercially available kit (RNEasy; No. 74104, Germantown, MD) was used to extract RNA from liver tissue and synthesize a cDNA library (Sigma, St. Louis, MO). A nanodrop spectrometer was utilized to assess the purity and quality of RNA and cDNA samples. Quantitative real-time PCR (qPCR) using an SYBR Green reagent (No. 172–5121, Bio-Rad Laboratories) and primer pairs (BRAND) were performed. Data are presented relative to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) using the $2^{-\Delta\Delta CT}$ method. See Table 2 for primer sequences.

Statistical Analysis

Kolmogorov-Smirnov and Shapiro-Wilk tests were conducted on all data before analysis to examine distribution of the data. Independent samples t-tests were used to determine differences in tissue weights, NAS, mRNA, and protein. Mann-Whitney nonparametric tests were used for data that were non-normally distributed, which includes R-βHB, locomotor activity (total, light, and dark), insulin sensitivity (insulin concentrations and HOMA-IR), mRNA expression of arginase 1 (*Arg1*)*, Il-1β, Col1A1,* monocyte chemoattractant protein-1 (*Mcp1*)*,* glucose 6-phosphatase (*G6Pase*)*,* and

GAPDH, steatosis, ballooning, inflammation, and protein content of PDGFβ, TRAIL, alpha-smooth muscle actin (αSMA), and CD11b. Mean weekly body weight and energy intake were analyzed using a 2 (group) x 10 (time) repeated-measures ANOVA. The Huynh-Feldt correction was used to report these interactions' significance due to violation of Mauchly's test of sphericity. Differences in lean body mass (LBM) and fat mass (FM) were analyzed by 2 (group) x 2 (time) repeated measures ANOVA. Group differences in REE and TEE (kcal/mouse/day) were determined by ANCOVA, with LBM and FM included as covariates as previously described[19,25,84,188]. All analyses were conducted with an n of 8 per group except for energy intake. During the first 2 weeks of the experimental phase of the study, food intake for two mice in the control group was eliminated due to a behavior pattern in which the mice removed all food from the feeding jar, which made it impossible to measure energy intake accurately. Significance was set *a priori* at $p < 0.05$, and data are expressed as means ± SD unless otherwise noted. All statistical analyses were conducted using IBM SPSS Statistics (version 29.0; IBM Corp., Armonk, New York).

CHAPTER 4: RESULTS

Dietary BD-$AcAc_2$ Attenuates Increases in Body Weight and Adiposity in the Presence of a HFHS Diet

There was a significant interaction between group and time on body weight (Fig. 4A; $p < 0.001$). Specifically, during weeks 1 through 9, mean body weight was significantly lower in KE compared to CON ($p < 0.05$). Between group differences in body weight were primarily attributed to a greater accretion of total adiposity in CON compared to KE (Fig. 4B; $p < 0.001$). Epididymal, retroperitoneal, and inguinal WAT weights confirm increased adiposity in the CON group, as the KE group accumulated significantly less visceral and subcutaneous fat (Fig. 4C; $p < 0.05$ for all). LBM increased in the CON group ($p < 0.001$) but was unchanged from baseline in the KE group ($p > 0.05$).

Effects of BD-$AcAc_2$ on Energy Intake and Energy Expenditure

There was a significant interaction between group and time on energy intake (Fig. 5A; $p = 0.007$). Mean energy intake was significantly lower in the KE group compared to CON ($p = 0.046$) one week after initiation of the diet. There were no significant differences in mean energy intake between groups in weeks 2 through 5. Beginning in week 6, mean energy intake was significantly higher in KE compared to CON ($p < 0.05$).

We also examined body weight-adjusted energy intake. There was a significant interaction between group and time on relative energy intake (Fig. 5B; $p = 0.010$). Specifically, beginning in week 4, mean relative energy intake was significantly higher in KE compared to CON ($p < 0.05$).

TEE and REE were significantly lower in KE compared to CON (Table 3; $p < 0.001$). However, there were no group differences in mean 24-hour TEE ($p = 0.610$) or REE ($p = 0.200$) after adjustment for FM and LBM. An analysis was conducted to further explore the data during the 12-hour dark cycle, when mice consume most of their energy, and during the 12-hour light cycle. Adjusted TEE and REE were not statistically different between groups for the dark or light cycle but were directionally higher in the KE-fed mice during the dark cycle and lower during the light cycle.

The KE group had a significantly higher 24-hour mean RER ($p = 0.024$) and dark cycle RER ($p = 0.002$) compared to CON (Fig. 6A). In contrast, RER during the light cycle was not significantly different between groups ($p = 0.314$). There were also no significant differences between groups in total, dark, or light locomotor activity (Fig. 6B; $p > 0.05$).

BD-$AcAc_2$ Attenuates Hepatic Steatosis, NAS, and Markers of HSC Activation and Fibrosis

The KE group had significantly lower hepatic steatosis, inflammation, ballooning, and total NAS compared to CON (Fig. 7A, 7B, and 7C, $p < 0.001$ for all). *αSma* ($p = 0.004$) and *Col1A1* ($p < 0.001$) mRNA expression, markers of collagen deposition and HSC activation, were significantly lower in KE compared to CON (Fig. 8A). However,

hepatic protein expression of the fibrogenesis markers, PDGFβ, matrix metalloproteinase 9 (MMP9), and matrix metalloproteinase 2 (MMP2), were not significantly different between groups ($p > 0.05$ for all, data not shown).

Effects of BD-$AcAc_2$ on Markers of Hepatic Inflammation

Cd68 mRNA expression ($p = 0.047$) and CD68 protein content ($p = 0.012$) were significantly lower in the KE group compared to CON (Fig. 8B and 8C). Hepatic pro-inflammatory M1 macrophage marker *Tnfα* ($p = 0.036$) and *Mcp1* ($p = 0.000$) mRNA expression were also lower in the KE group compared to CON. There were no significant differences between groups in hepatic pro-inflammatory M1 *Il-1β* mRNA expression or IL-1β, TRAIL, or CD11b protein content ($p > 0.05$). mRNA expression of *Cd163* was higher in the KE group compared to CON but did not achieve statistical significance (Fig. 8B; $p = 0.062$). However, CD163 protein expression was significantly higher in the KE group compared to CON (Fig. 8C; $p = 0.004$). No statistically significant differences were observed between groups for other M2 markers, *Arg1* and mannose receptor c-type 1 (*Mrc*1) (Fig. 8B; $p > 0.05$).

Exploratory Analysis of DNL, Mitochondrial Biogenic Markers, and Glucose Metabolism

The present investigation was a proof-of-concept study designed to test our hypothesis that BD-$AcAc_2$ would attenuate hepatic lipid accumulation and inflammation on a HFHS diet. The phenotype observed here and in our previous studies [19,25,29] highlights the need to examine potential molecular mechanisms. Thus, we examined

mRNA expression of key components of DNL to explore whether lower liver lipid content in the KE group was due to decreased DNL expression. Hepatic protein content of acetyl CoA carboxylase (ACC) and fatty acid synthase (FASN) were not significantly different between groups (Fig. 9A; $p > 0.05$ for both). mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*Pgc1-α*; $p = 0.796$) and mitochondrial transcription factor a (*Tfam*; $p = 0.716$) (Fig. 9B) were also not significantly different between groups, suggesting that hepatic markers of mitochondrial biogenesis were unaltered by BD-$AcAc_2$ administration.

R-βHB concentrations were higher in the KE group compared to CON (Table 4). S-βHB and AcAc concentrations that require more sophisticated experimental approaches were not measured and likely contribute to a significant underestimation of total circulating ketones. Serum glucose and insulin concentrations were measured to examine effects of the ketone ester on glucose metabolism. Glucose ($p = 0.371$) did not reach statistical significance for differences between groups. However, circulating concentrations of insulin were significantly lower in KE compared to CON (Table 4) ($p = 0.038$). QUICKI scores were significantly higher in the KE group than in the CON ($p = 0.026$), while HOMA-IR was significantly lower ($p = 0.021$). mRNA expression of *Pepck* ($p=0.343$) and *G6pase* ($p = 0.381$) were not significantly different between groups (Fig. 9C), providing initial evidence that a reduction in hepatic gluconeogenesis may not contribute to the improvement in markers of insulin sensitivity.

CHAPTER 5: DISCUSSION

Previous studies show that the ketone ester, BD-$AcAc_2$, reduces[25,189] or lowers the accretion of[19,26,27,84] body weight and adiposity under various dietary and environmental conditions and rodent characteristics. A more recent publication from our group showed that BD-$AcAc_2$ decreased hepatic steatosis, fibrosis, and inflammatory markers compared to *ad libitum* HFD-fed obese controls and pair-fed animals receiving similar energy provisions as ketone-treated animals (~26% less energy than control-fed animals)[29]. In each of these studies, 25-30% of carbohydrate energy was removed to produce isocaloric diets[19,25,29,84]. However, it was speculated that the reported responses could be partially explained by the metabolic effects of reducing dietary carbohydrates. Therefore, the purpose of this investigation was to examine whether adding BD-$AcAc_2$ to the diet without removing carbohydrate energy in the presence of a HFHS diet would attenuate the accretion of adiposity and markers of NAFLD. Our findings indicate that ketone-fed mice accrued less adiposity than controls and had lower NAS (inflammation, steatosis, and ballooning), fibrosis, and HSC activation.

HFHS "Western" diets produce positive energy balance leading to obesity and obesity-related metabolic diseases such as NAFLD. In the current investigation, mice in the CON group developed a phenotype consistent with NAFLD. However, mice in the KE group gained only a fraction of the body weight and adiposity observed in the CON

group, indicating that BD-$AcAc_2$ produced an effect on one or more components of energy balance. Despite significant differences in body weight and adiposity, absolute energy intake was similar between groups. While reporting energy intake relative to body weight may not be appropriate due to differences in energy requirements of various tissues[190], a 22% higher relative energy intake suggests that energy needs were higher in the KE group to maintain body composition. Previous studies in which carbohydrate energy was removed to accommodate energy from the dietary ketone show that energy intake is either decreased[19,25] or unchanged[19,84]. Davis and colleagues[25] reported that BD-$AcAc_2$-fed obese mice on a HFD experienced a 26% decrease in energy intake. However, mice in the KE group had significantly lower body weight and adiposity than a pair-fed group despite receiving similar energy provisions. In contrast, lean mice placed on a LFD with BD-$AcAc_2$ administration had similar absolute and body-weight relative energy intake compared to controls[19]. These findings and those from the current investigation indicate that a decrease in energy intake and, for the first time, removal of carbohydrate energy (in the presence of a HFHS diet) is unnecessary to limit the accretion of adiposity. While the mechanisms responsible for the robust differences in body weight and adiposity observed with mice receiving the ketone diester remain elusive, we predict that energy expenditure is likely higher during the transition to ketone diester administration. By measuring energy expenditure at the completion of the study in each of our previous investigations, including this one, we are likely missing early changes in components of energy intake, energy expenditure, and energy loss. Upcoming studies are planned to thoroughly interrogate early responses that are likely contributing to the body composition phenotype.

Adjusted 24-hour TEE and REE were similar between groups when measured at the completion of the study. While dark cycle TEE and REE were not statistically different between groups, the KE group had a noticeable pattern of higher dark cycle TEE and REE, which is when mice consume over 70% of their daily energy. In contrast, mice in the KE group had statistically similar but numerically lower TEE and REE during the light cycle than the CON group. BD-$AcAc_2$-fed obese mice on a HFD had significantly higher TEE and REE compared to pair-fed animals receiving similar energy provisions suggesting that increased energy expenditure contributed to development of the phenotype[25]. As part of a LFD, BD-$AcAc_2$ produced no change or a slight decrease in energy expenditure[19,84]. In our previous investigation[25], we observed a transcriptional signature in BAT, but not WAT, that was consistent with a thermogenic phenotype. Although it was not within the scope of work in this investigation, future studies are needed to explore the possibility that increasing circulating ketones with exogenous ketone esters could increase thermogenic capacity in BAT.

Ketone energy losses through breath, urine, and feces are challenging to measure in rodents and the current nutritional model, where ketones are incorporated into the diet and consumed at each feeding rather than at designated times. Conclusions drawn about the results would assume that the timing of measurements would reflect energy loss throughout exposure to the ketone supplement, which may be misleading. A previous investigation measured feces output while on a LFD and found that ketone-fed mice had 50% greater fecal loss[84]. However, fecal energy loss explained only 25% of the difference in adiposity between groups[84]. Although we did not collect feces in the current

investigation, it will be important to establish the contribution of energy loss in future studies to define the mechanisms responsible for the body weight phenotype produced.

A limitation of the current investigation is that we measured energy expenditure only during the final week of the study. Assessing energy expenditure at that point, when the gap in body weight is so great, may contribute to the absence of an appreciable signal to indicate that an increase in energy expenditure occurred. The CON group's significantly higher body weight and adiposity than the KE group require statistical adjustment using FM and LBM for comparison purposes. Future studies should focus on changes in energy expenditure during the introduction of the diet before appreciable differences emerge in body mass to determine the role of BD-$AcAc_2$ on energy balance. Conducting these studies will help to reveal whether a difference in energy expenditure or energy loss exists and, if so, which component of energy expenditure is the most affected. It will also be important to devise methods to measure energy losses through breath and urine, as these likely contribute to the phenotype and may affect indirect calorimetry results[191]. Finally, our studies to date have only included male mice. Considering that female mice may respond differently to the diet regarding the accretion of adiposity and hepatic lipid accumulation than male mice, future studies are planned by our group to investigate sexual dimorphism using this nutritional model.

Excess energy and obesity are strongly associated with ectopic fat accumulation in humans[192] and rodents[193], including the pancreas, kidney, heart, skeletal muscle, and liver. NAFLD is considered the hepatic manifestation of obesity and metabolic syndrome and can progress to nonalcoholic steatohepatitis (NASH), liver fibrosis, and cirrhosis[169]. Currently, there are no effective pharmacological approaches to treat NAFLD, with

lifestyle modifications (i.e., diet and exercise) serving as the primary form of treatment. Despite the short-term superiority of KD, in the long-term, LFD appears to produce similar reductions in liver fat if a threshold of weight loss is achieved[169,194].

We previously reported that BD-$AcAc_2$ improved markers of NAFLD in HFD-fed obese mice compared to both control and pair-fed mice[29]. In the present investigation, BD-$AcAc_2$ abolished the development of hepatic steatosis, inflammation, and ballooning in lean mice compared to controls on an identical HFHS diet. These results provide evidence that BD-$AcAc_2$ decreases markers of NAFLD in obese mice on a HFD and lean mice placed on a HFHS diet despite similar energy intake and expenditure between groups. These findings are potentially important as they suggest mechanisms independent of CR or reductions in carbohydrate energy are responsible for the improved hepatic outcomes, which could have important clinical implications. BD-$AcAc_2$ may also protect against HSC activation and fibrosis as mRNA expression of *αSma* and *Col1A1*, markers of collagen deposition, were significantly lower in the KE group compared to the CON group. This finding is critical because HSC activation is a distinguishing trait of NAFLD progression and indicates a fibrogenic phenotype[195].

Another hallmark of the progression of NAFLD to NASH is hepatic inflammation, specifically, an imbalance in pro- and anti-inflammatory macrophages[183]. Ketone administration reduces inflammation in multiple tissues, such as the heart, kidney, and liver[43,68,69]. Indeed, we demonstrated that BD-$AcAc_2$ lowered the expression of multiple pro-inflammatory M1 markers and higher expression of M2 anti-inflammatory markers. Mitigation of hepatic inflammation in ketone-fed mice may be due to the anti-inflammatory properties of βHB. The anti-inflammatory effects of βHB appear to be at

least partially mediated by inhibition of the NLRP3 inflammasome[43]. In a mouse model of lipopolysaccharide (LPS)-induced sepsis, mice fed a ketone monoester had reduced markers of NLRP3 activation and markers of hepatic inflammation, including *Il-1β* and *Tnfα*[43]. Others[42] show that both R- and S-enantiomers of βHB (but not AcAc) inhibit the NLRP3 inflammasome by preventing K^+ efflux and reducing ACS oligomerization and speck formation in a GPR109A (HCAR2) and histone deacetylase (HDAC) independent fashion in macrophage cell culture. In the same investigation, βHB also attenuated caspase-1 activation and IL-1β secretion in mouse models of NLRP3-mediated disease. These findings, taken together with our own, suggest that βHB-mediated inhibition of the NLRP3 inflammasome may play a role in the hepatic anti-inflammatory effects of BD-$AcAc_2$. However, additional work is needed using *in vitro* and *in vivo* NLRP3 inflammasome loss of function models. Others[179] have shown that exogenous AcAc, but not βHB, ameliorated diet-induced hepatic fibrosis through mechanisms that appear linked to the oxidation of AcAc in macrophages, as evidenced by the observation that SCOT null mice (which are unable to oxidize AcAc) had increased fibrosis. These findings suggest that AcAc or βHB-mediated inhibition of inflammatory pathways may play a role in the anti-inflammatory effects of BD-$AcAc_2$.

Diets high in added sugars, such as sucrose, often upregulate the expression and activity of proteins involved in DNL[43]. Therefore, we measured protein expression of ACC and FASN, which are well-known markers of DNL activity. In the current investigation, BD-$AcAc_2$ did not affect hepatic protein content of either ACC or FASN. More sophisticated labeling approaches using heavy water and mass spectrometry are needed, as transcriptional levels of key enzymes in the DNL cascade do not always

translate into changes in enzyme activity. The HFHS diet used in this investigation would be expected to reduce endogenous ketogenesis by reducing the rate-limiting enzyme in ketogenesis, HMGCS2[196]. Reductions in HMGCS2 expression have been shown to increase DNL in mature mice on a HFD by eliminating the capacity to siphon carbons from the TCA cycle toward ketogenesis. It is possible that exogenous ketones re-activate this pathway through mechanisms that could involve activation of HDACs. We also did not find that markers of mitochondrial biogenesis were increased in the liver. However, future studies should examine mitochondrial respiration in BAT, skeletal muscle, and liver to determine if the ketone ester alters coupled and/or uncoupled respiration.

The observations in liver may also be attributed to the BD-$AcAc_2$'s ability to attenuate the accretion of adiposity on a HFHS diet, thereby limiting ectopic deposition or production of liver fat. A close examination of the data in the current study provides evidence that an attenuation in body weight and adiposity is not required to mitigate the increase in hepatic lipid content observed in control animals. For example, one mouse in the KE group gained a similar amount of body weight and adiposity to the relatively uniform increases observed in CON mice. However, this animal's liver size and weight were less than those observed in CON animals and had a NAS of 2.0 (steatosis 2; inflammation 0; ballooning 0), compared to the CON group, which had a mean NAS of 7.1 (steatosis 3.0; inflammation 2.1; ballooning 2.0). These findings suggest that hepatic outcomes may be improved by BD-$AcAc_2$ in a HFHS setting regardless of weight or adiposity status. Future studies are planned to determine whether BC-$AcAc_2$ directly affects hepatic lipid metabolism and inflammation.

While circulating R-βHB concentrations were significantly higher in the KE group compared to the CON group, the measured ketone concentrations may not reflect the true level of ketosis reached throughout the experiment with the ketone ester. First, a carefully conducted fasting and re-feeding protocol was not followed, which would be necessary to control the timing of the last feeding bout more tightly. In addition, current hand-held technology does not detect S-βHB and AcAc concentrations from whole blood, and more-sophisticated approaches using expensive tandem mass spectrometry procedures were outside the scope of the current investigation. This was also not a primary outcome measure for the study, as it has been demonstrated that the ketone ester increases βHB and AcAc concentrations in rodents[25-28,84,189].

Circulating insulin concentrations were significantly lower, and QUICKI and HOMA-IR calculations indicate greater insulin sensitivity in the KE-fed mice. These findings are important because reductions in circulating insulin could contribute to increased adipose tissue lipolysis and fatty acid oxidation. However, there were no differences in glucose concentrations between groups, indicating that the level to which BD-$AcAc_2$ affects insulin and glucose homeostasis is also affected by the carbohydrate content of the diet. While mice were not fasted overnight in the present investigation, human studies show that fasting and non-fasting estimates of insulin sensitivity using QUICKI and HOMA-IR are moderately correlated[197]. Other approaches, such as hyperinsulinemic-euglycemic clamp with tracers, will be needed to examine whether the ketone ester improves insulin sensitivity and in what tissue(s). Although our initial findings suggest that BD-$AcAc_2$ administration does not alter markers of

gluconeogenesis, it will be important for future studies using tracers to fully examine hepatic glucose production.

The findings of this study, while challenging to explain mechanistically, have significant potential from a clinical and practical perspective. CR-mediated weight loss is the most used strategy to reduce NAFLD in humans and rodents. The downside is that CR is often ineffective long-term, with weight regain and metabolic dysfunction returning within the first 2-5 years[124]. By adapting the results from our current and previous[29] investigations to humans, we could avoid the strict requirements of CR and KD. Another fascinating observation that will require additional work is that most of the weight gain in the KE-fed mice was due to a non-statistically significant increase in LBM. A recent publication from our laboratory showed that in older mice, several markers of skeletal muscle hypertrophy and atrophy are altered by BD-$AcAc_2$ in a pattern suggestive of improvements in skeletal muscle quantity and quality[198].

Final Conclusion

These findings are the first to demonstrate that the dietary ketone ester, BD-$AcAc_2$, attenuates weight and body fat gain in C57BL6 mice on a HFHS diet. Previous investigations examined the effects of exogenous ketones with dietary carbohydrates removed to produce isocaloric control diets. Current findings show that adding the ketone ester to the diet without removing carbohydrate energy (i.e., in the presence of high fat and high sugar content) prevented increases in body weight and adiposity. The mechanisms responsible for attenuating weight gain are unclear but appear to stem from a combination of energy expenditure and energy loss, although not examined in this study.

Furthermore, BD-$AcAc_2$ completely abolished liver steatosis and markers of fibrosis and inflammation through mechanisms that further exploration.

Currently, there are no approved pharmaceutical treatment strategies for NAFLD. With the limited long-term capacity of dietary and lifestyle modifications, novel approaches are needed to reduce the development and progression of NAFLD. Exogenous ketones may provide a new approach for treating NAFLD and obesity, and our long-term objective is to test this hypothesis in humans. Based on our findings and others, it has been shown that whole-body glucose metabolism was improved in mice administered a ketone diester or monoester. This suggests that exogenous ketones could present a treatment strategy to improve glucose control and insulin resistance in individuals with obesity-related metabolic conditions, such as T2DM. Studies are planned to examine in parallel the varying short- and long-term effects of a ketone monoester vs. the ketone diester used in this investigation. These studies will provide valuable information on the metabolism of AcAc, R-βHB, and S-βHB and the plausible loss of energy accompanying its availability in the ester. Overall, the results of the current and planned investigations will contribute to our understanding of the capacity to which exogenous ketone administration prevents the development of phenotypes consistent with obesity and NAFLD, as well as to our ability to translate these findings to humans.

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FIGURE LEGENDS

Fig 1. Overview of ketogenic pathway. Ketones are produced within hepatic mitochondria from fatty acid derived acetyl CoA. AcAc and βHB are exported to extraheptic tissues for terminal oxidation. Abbreviations: AcAc, acetoacetate; βHB, β-hydroxybutyrate

Fig 2. Metabolic fates of ketone bodies. Ketone bodies produced in the liver are 1) excreted in the urine and breath, 2) diverted to lipogenesis or cholesterol synthesis, or 3) catabolized in the mitochondria of extrahepatic tissues to acetyl-CoA, which can be used in the TCA cycle. Abbreviations: TCA cycle, tricarboxylic acid cycle

Fig 3. Metabolism of ketone monoester vs. ketone diester. 3A) R-3-hydroxybutyrate-R-1,3-butanediol is hydrolyzed to R-βHB and R-1,3-butanediol by esterases found in the gastrointestinal tract, blood, and liver. R-1,3-butanediol is converted to D-βHB and AcAc in the liver by alcohol and aldehyde dehydrogenases. 3B) R,S-1,3-butanediol diacetoacetate is hydrolyzed to butanediol and AcAc by esterases in plasma and tissues. R,S-1,3-butanediol undergoes hepatic oxidation to both the R- and S-isoforms of βHB. Abbreviations: AcAc, acetoacetate; Alc DH, alcohol dehydrogenase; Ald DH, aldehyde dehydrogenase; βHB, β-hydroxybutyrate;

Fig. 4. Differences in body weight, body composition, and tissue weights after 9 weeks of CON (HFHS) or KE (HFHS + 25% ketone ester by kcals) diets. 4A) Body weight responses across 9 weeks. *$p < 0.05$, CON vs. KE beginning at week 1. 4B) Pre- and Post-FM and LBM measurements. *$p < 0.001$. FM gain in the CON group was significantly greater than the KE group. *$p <0.001$. 4C) Final tissue weights for EWAT, RPWAT, and IWAT, BAT, liver, kidney, heart, and VL. *$p < 0.05$ for all adipose tissue weights and liver weight. Values are presented as means ± SD. Abbreviations: CON, control; EWAT, epididymal white adipose tissue; HFHS, high-fat, high-sugar; IWAT, inguinal white adipose tissue; KE, ketone ester; RPWAT, retroperitoneal white adipose tissue

Fig. 5. Differences in energy intake after 9 weeks of CON (HFHS) or KE (HFHS + 25% ketone ester by kcals) diets. 5A) Energy intake in kcal/mouse/d. *$p < 0.001$ at week 1; $p > 0.05$ for the remainder of the experimental phase. 5B) Energy intake in kcal/g BW. *$p < 0.05$ beginning at week 4 and for the remainder of the experimental phase. Values are presented as means ± SD. Abbreviations: CON, control; HFHS, high-fat, high-sugar; kcal/g BW, kilocalorie per gram body weight; kcal/mouse/d, kilocalorie per mouse per day; KE, ketone ester

Fig. 6. Measurement of final RER and physical activity counts. 6A) RER was calculated as VCO_2/VO_2. $p < 0.05$, total and dark RER. 6B) Physical activity counts were measured using XT and YT activity. Values are presented as means ± SD. $p < 0.05$, * indicates significant differences between groups. Abbreviations: RER, respiratory exchange ratio; VCO_2, volume of carbon dioxide; VO_2, volume of oxygen

Fig. 7. Differences in liver phenotype assessed at study completion. 7A and B) Representative hematoxylin and eosin images of liver morphology. CON group has greater accumulation of lipids compared to KE. 7C) Differences in NAS (fat accumulation, inflammation, and hepatocellular ballooning), calculated by grading and scoring liver specimens. Values are presented as means ± SD. $p < 0.05$, * indicates significant differences between groups. Abbreviations: CON, control; KE, ketone ester; NAS, nonalcoholic fatty liver disease activity score

Fig. 8. Markers of hepatic stellate cell activation, fibrosis, and hepatic inflammation after 9 weeks of CON or KE diets. 8A) Hepatic markers of fibrosis, *αSma* and *Col1A1* mRNA expression. 8B) mRNA expression of inflammatory hepatic marker *Cd68*, pro-inflammatory hepatic M1 markers *Il-1β, Tnfα,* and *Mcp1*, and anti-inflammatory hepatic M2 markers *Cd163, Arg1*, and *Mrc1*. 8C) Protein content of inflammatory hepatic marker CD68, pro-inflammatory hepatic M1 markers Il-1β and CD11b, and anti-inflammatory hepatic M2 marker Cd163. Values are presented as means ± SD. $p < 0.05$, * indicates significant differences between groups. Abbreviations: *Arg1,* arginase 1; CD11b, cluster of differentiation 11b; *Cd163,* cluster of differentiation 163; *Col1A1*, collagen 1 A1; CON, control; *Il-1β*, interleukin-1β; KE, ketone ester; *Mcp1,* monocyte chemoattractant protein-1; *Mrc1,* mannose receptor c-type 1; RQ, relative quantification; *Tnfα*, tumor necrosis factor α; *αSma*, α-smooth muscle actin

Fig. 9. Markers of DNL and mitochondrial biogenesis. 9A) Protein content of hepatic ACC and FASN. 9B) mRNA expression of key components of DNL, *Pgc1-α* and *Tfam*. 9C) mRNA expression of markers of gluconeogenesis*, Pepck* and *G6pase*. Values are presented as means ± SD. $P < 0.05$, * indicates significant differences between groups. Abbreviations: ACC, acetyl CoA carboxylase; DNL, *de novo* lipogenesis; FASN, fatty acid synthase; *G6pase,* glucose 6-phosphatase*;Pepck,* phosphoenolpyruvate carboxykinase; *Pgc1-α,* peroxisome proliferator-activated receptor gamma coactivator 1-*α;* RQ, relative quantification; *Tfam*, mitochondrial transcription factor a

Table 1. Composition of high-fat high-sugar control (CON) and ketone ester (KE) diets

Ingredient	kcal/g	CON (g/kg)	KE (g/kg)	CON (kcal/kg)	KE (kcal/kg)
Casein	3.6	250.0	250.0	895.0	895.0
L-Cystine	4.0	3.0	3.0	12.0	12.0
Sucrose	4.0	440.0	440.0	1760.0	1760.0
Lard	9.0	200.0	200.0	1800.0	1800.0
Cellulose	0.0	49.5	49.5	0.0	0.0
Mineral Mix #210025	0.9	35.0	35.0	30.8	30.8
Vitamin Mix #310025	3.9	10.0	20.0	38.7	38.7
Choline Bitartrate	0.0	2.5	2.5	0.0	0.0
Sodium Saccharin	0.0	10.0	10.0	0.0	0.0
Ketone Ester	5.8	0.0	195.6	0.0	1134.3
Total		1000.0	1195.6	4537.0	5671.0

Table 2. Primer Sequences

Gene Name	Forward (5'-3')	Reverse (3'-5')
CD68	CAATTCAGGGTGGAAGAAAG	TCTGATGTAGGTCCTGTTTG
CD163	AGTCTGCTCACGATACATAG	TCCTTCTGGAATAGATTGGG
GAPDH	CTTCAACAGCAACTCCCACTC	GCCGTATTCATTGTCATACCAGG
G6Pase	AGGAAGGATGGAGGAAGGAA	TGGAACCAGATGGGAAAGAG
MCP-1	AGCTGTAGTTTTTGTCACCAAGC	GTGCTGAAGACCTTAGGGCA
PEPCK	CCACAGCTGCTGCAGAACAC	GAAGGGTCGCATGGCAAA
TNFα	GTGACAAGCCTGTAGCCCAC	GCAGCCTTGTCCCTTGAAGA

Table 3. Resting and Total Energy Expenditure

	Daily (kcal)		Dark Cycle (kcal/h)		Light Cycle (kcal/hr)	
	REE	TEE	REE	TEE	REE	TEE
CON						
Unadjusted	9.8 ± 0.9^{a}	12.8 ± 1.0^{a}	0.44 ± 0.04^{a}	0.56 ± 0.04^{a}	0.42 ± 0.04^{a}	0.50 ± 0.04^{a}
Adjusted	9.5 ± 0.4^{a}	12.2 ± 0.3^{a}	0.42 ± 0.02^{a}	0.53 ± 0.01^{a}	0.40 ± 0.02^{a}	0.48 ± 0.02^{a}
KE						
Unadjusted	8.3 ± 0.5^{b}	11.2 ± 0.5^{b}	0.41 ± 0.03^{b}	0.51 ± 0.03^{b}	0.35 ± 0.02^{b}	0.42 ± 0.02^{b}
Adjusted	8.6 ± 0.4^{a}	11.9 ± 0.3^{a}	0.43 ± 0.02^{a}	0.55 ± 0.01^{a}	0.36 ± 0.02^{a}	0.43 ± 0.02^{a}

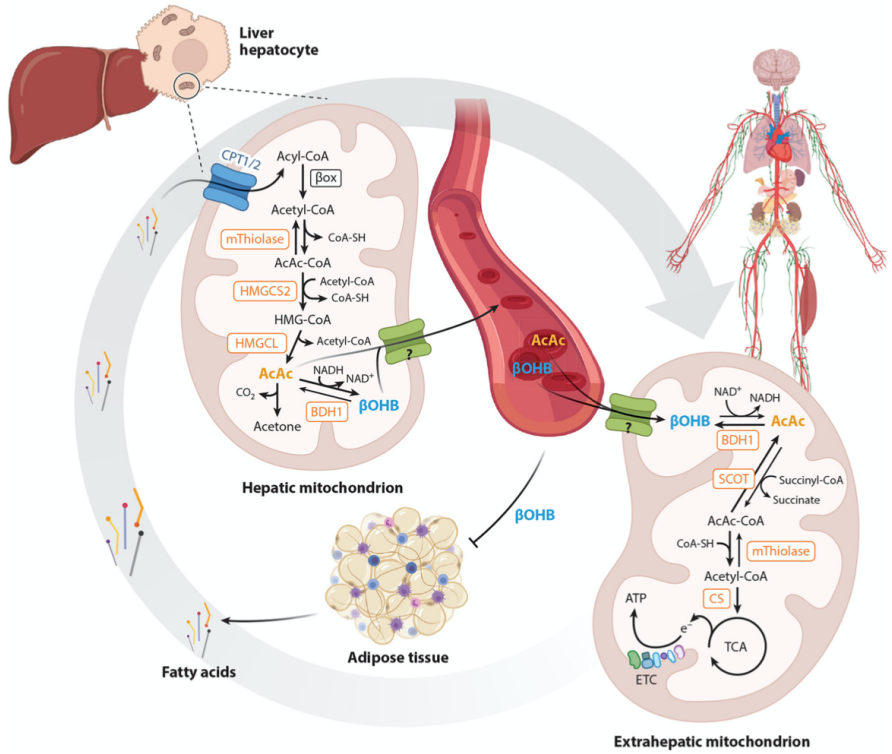
*Values were adjusted for Fat Mass (FM) and Lean Body Mass (LBM) using ANCOVA. Values are represented as means ± SD from 24-hour or 12-hour (dark and light cycle) continuous indirect calorimetry performed following the 9-week intervention. Mice were acclimated in the TSE system for 48 hours before gases were measured for 24 hours. Values with different letters are significantly different ($p < 0.05$).

Table 4. Blood Markers

	CON	KE
Insulin ($ng \cdot mL^{-1}$)	4.3 ± 2.8	2.3 ± 1.1*
Glucose ($mg \cdot dL^{-1}$)	215 ± 35	202 ± 22
NEFA ($nmol \cdot \mu L^{-1}$)	31.5 ± 6.6	38.4 ± 7.8
R-βHB (mM)	0.1 + 0.0	0.338 + 0.3

*Values are means ± SD. * = significance set at $p < 0.05$.

NEFA = Non-esterified fatty acids



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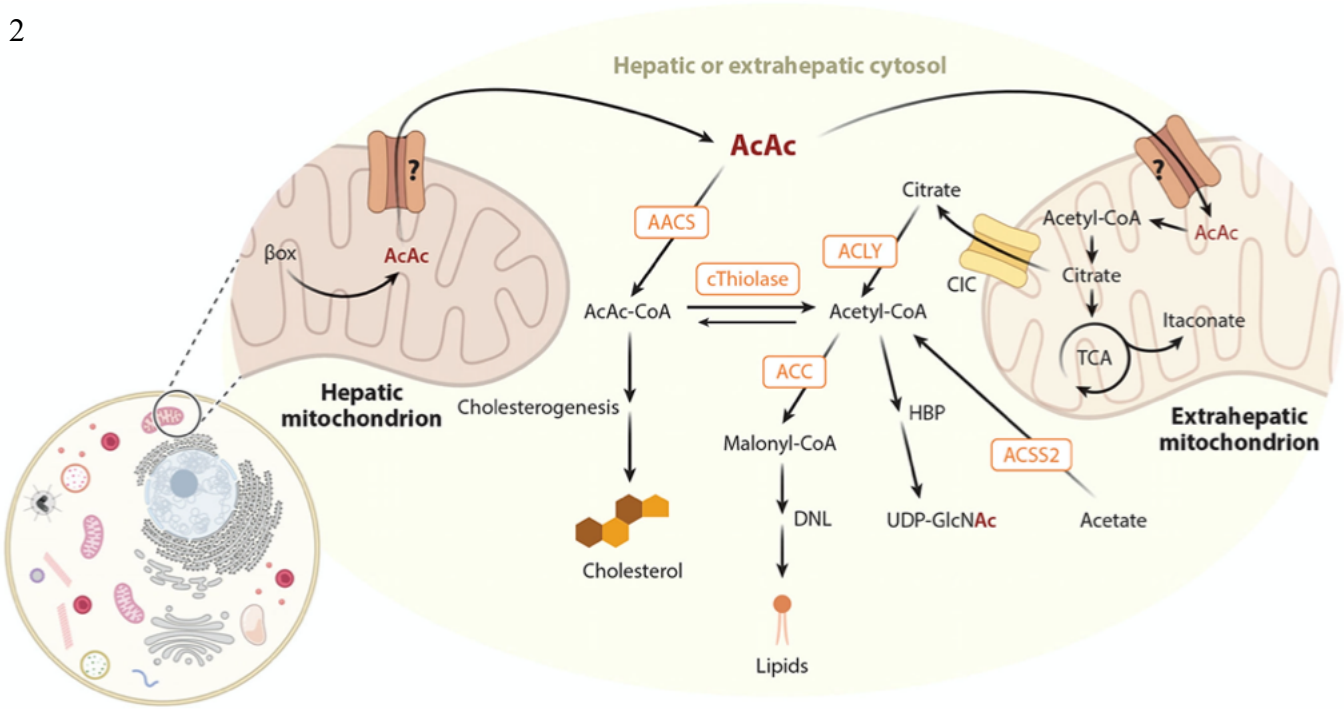
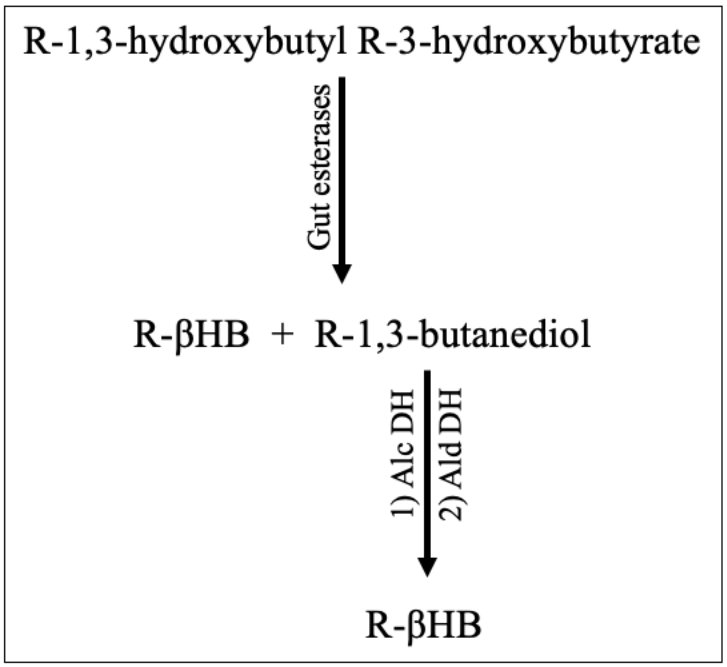
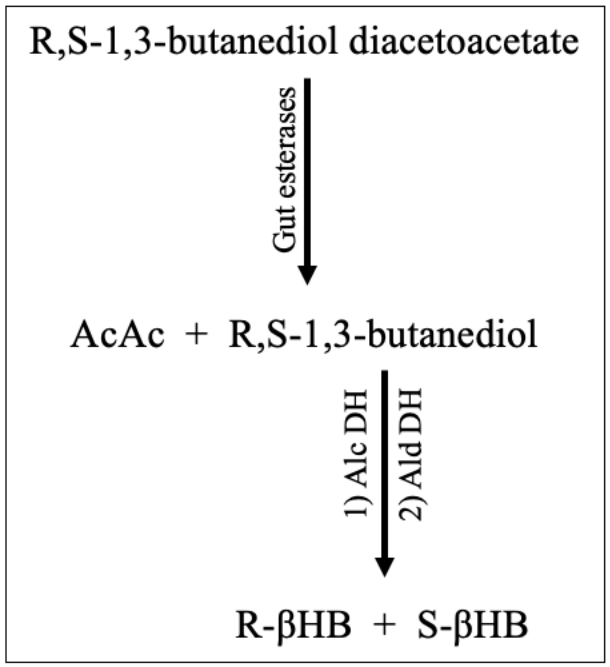


Puchalska P, *Annu Rev Nutr,* 2021

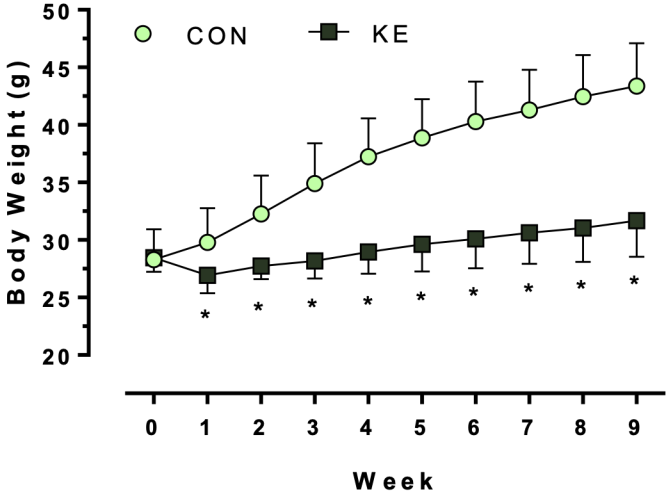
3A


R-1,3-hydroxybutyl R-3-hydroxybutyrate
Gut esterases
R-βHB + R-1,3-butanediol
1) Alc DH
2) Ald DH
R-βHB

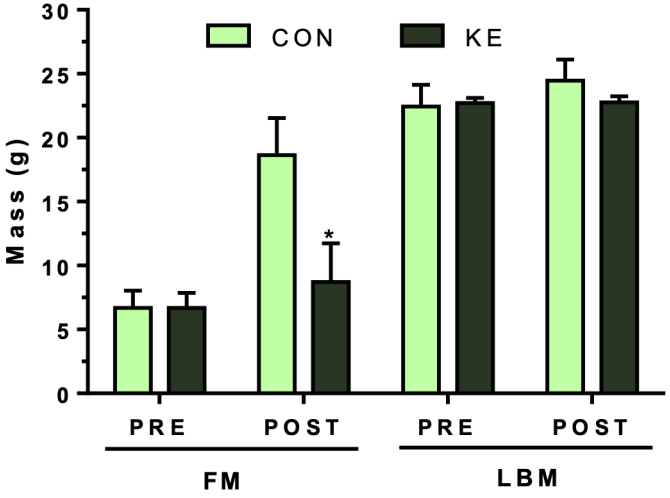
3B


R,S-1,3-butanediol diacetoacetate
Gut esterases
AcAc + R,S-1,3-butanediol
1) Alc DH
2) Ald DH
R-βHB + S-βHB

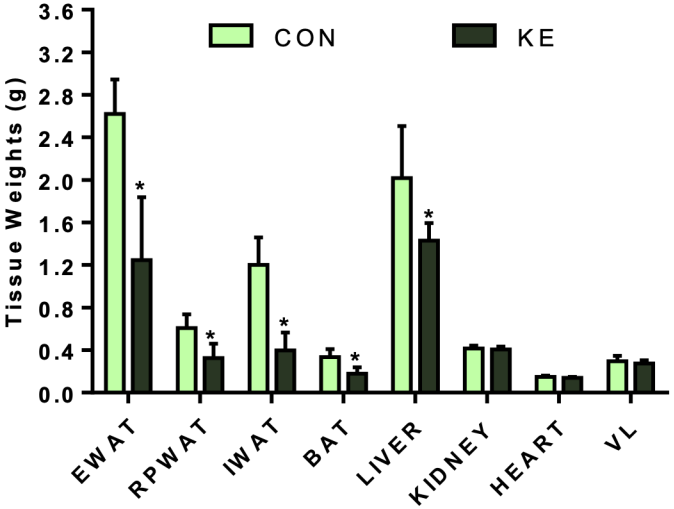
4A


CON
KE
Body Weight (g)
50
45
40
35
30
25
20
0 1 2 3 4 5 6 7 8 9
Week

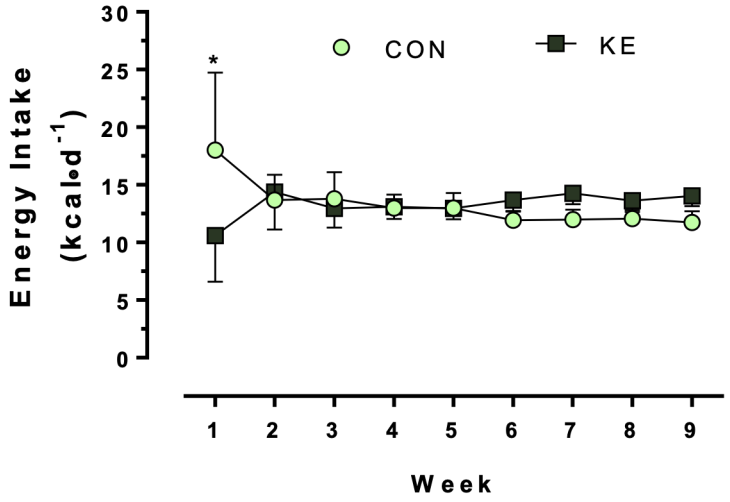
4B


CON
KE
Mass (g)
30
25
20
15
10
5
0
PRE
POST
PRE
POST
FM
LBM

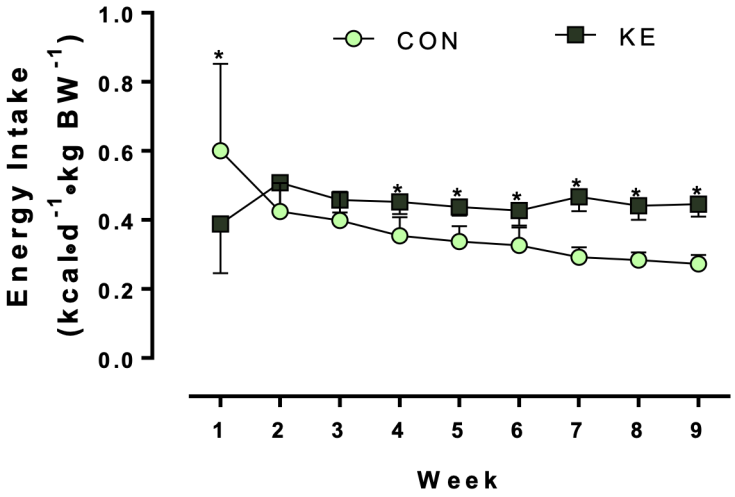
4C


CON
KE
Tissue Weights (g)
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0.0
EWAT
RPWAT
IWAT
BAT
LIVER
KIDNEY
HEART
VL

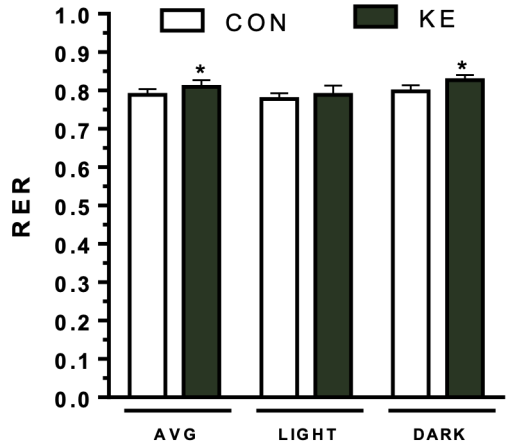
5A


Energy Intake
(kcal•d-1)
CON
KE
Week
0
5
10
15
20
25
30
1
2
3
4
5
6
7
8
9
*

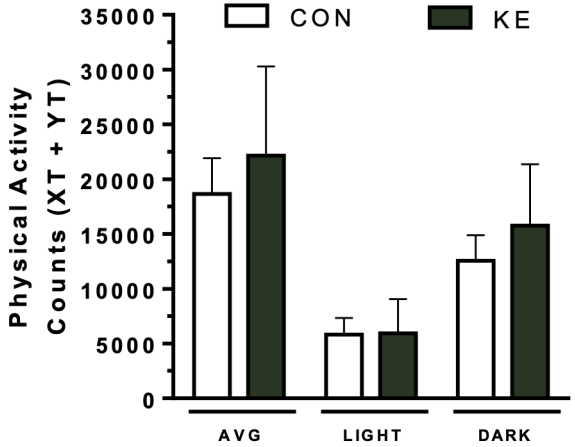
5B


Energy Intake
(kcal•d-1•kg BW-1)
CON
KE
Week
0.0
0.2
0.4
0.6
0.8
1.0
1
2
3
4
5
6
7
8
9
*

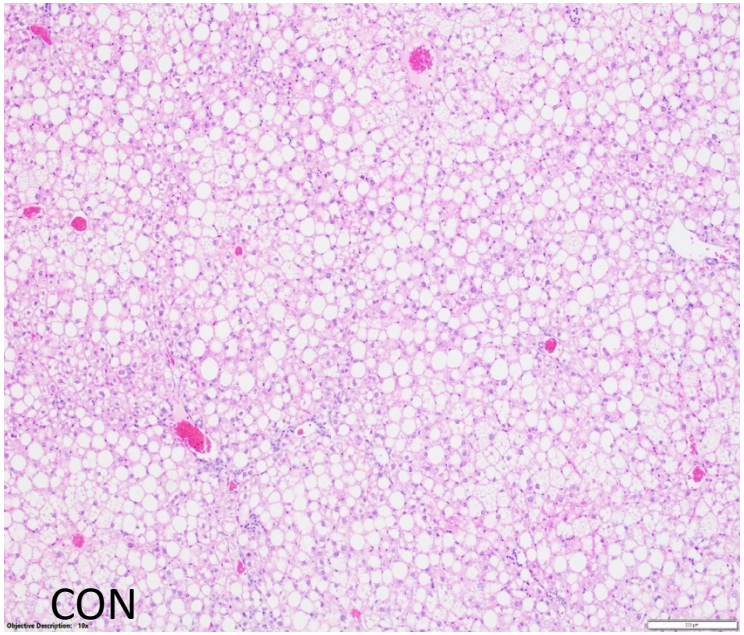
6A


CON
KE
RER
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
AVG
LIGHT
DARK

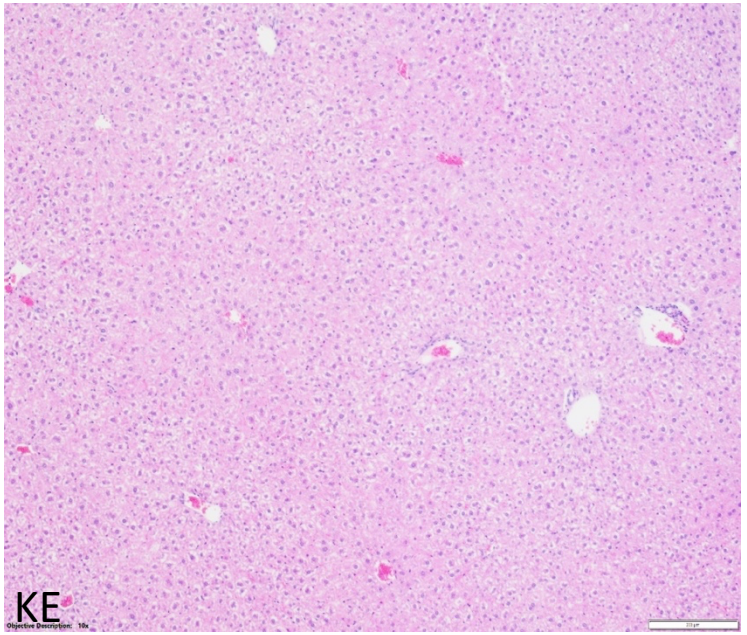
6B


CON
KE
Physical Activity
Counts (XT + YT)
35000
30000
25000
20000
15000
10000
5000
0
AVG
LIGHT
DARK

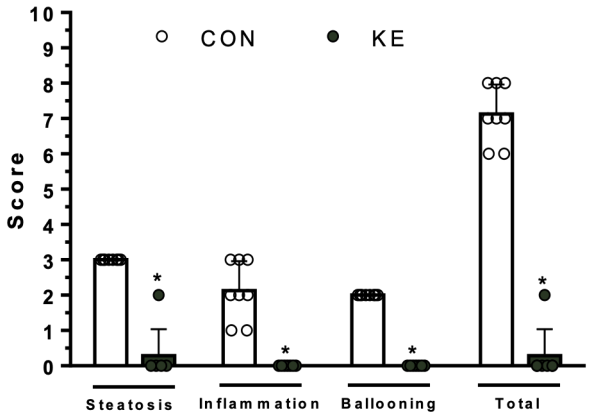
7A


CON

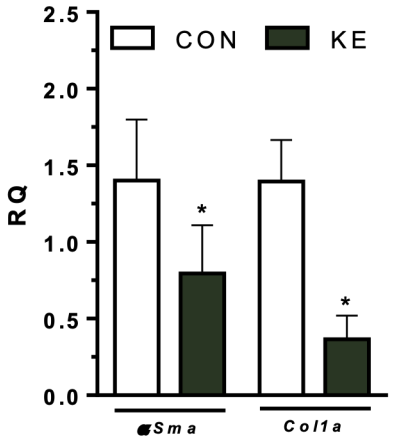
7B


KE

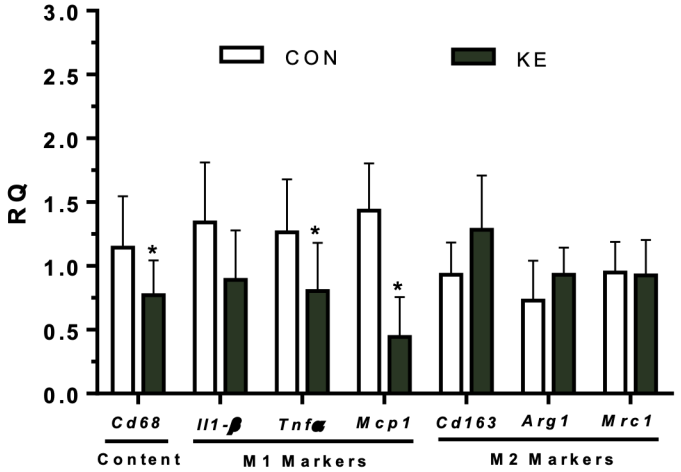
7C


CON
KE
Score
10
9
8
7
6
5
4
3
2
1
0
*
*
*
*
Steatosis
Inflammation
Ballooning
Total

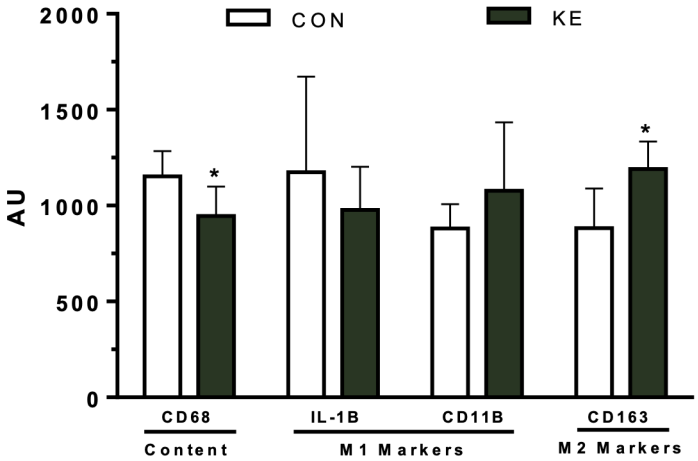
8A


CON
KE
RQ
2.5
2.0
1.5
1.0
0.5
0.0
*
*
αSma
Col1a

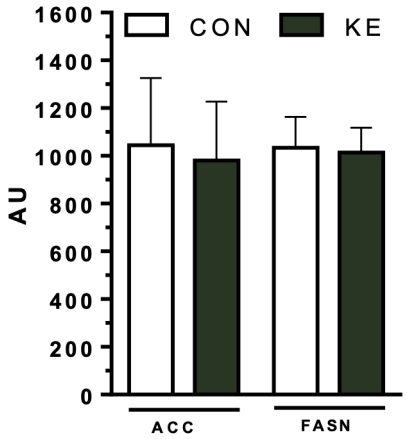
8B


CON
KE
RQ
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Cd68
Il1-β
Tnfα
Mcp1
Cd163
Arg1
Mrc1
Content
M1 Markers
M2 Markers

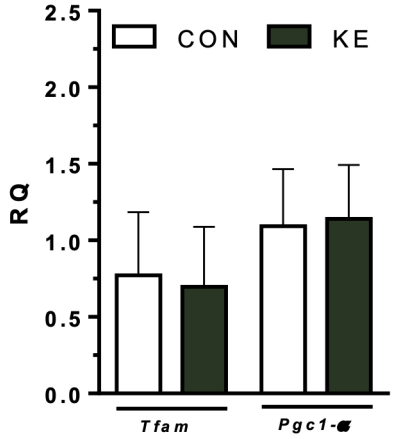
8C


CON
KE
AU
2000
1500
1000
500
0
CD68
IL-1B
CD11B
CD163
Content
M1 Markers
M2 Markers

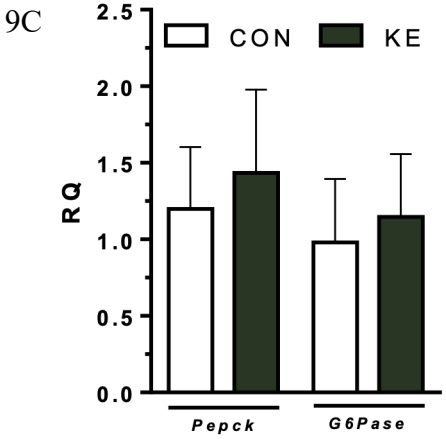
9A


CON
KE
AU
1600
1400
1200
1000
800
600
400
200
0
ACC
FASN

9B


CON
KE
RQ
2.5
2.0
1.5
1.0
0.5
0.0
Tfam
Pgc1-α

9C


CON
KE
RQ
2.5
2.0
1.5
1.0
0.5
0.0
Pepck
G6Pase