

# Much Ado About Eating: Dietary Therapy for Health and Disease Management

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Boston College  
The Graduate School of Arts and Sciences  
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MUCH ADO ABOUT EATING: DIETARY THERAPY FOR  
HEALTH AND DISEASE MANAGEMENT

A dissertation

By

JOSHUA J. MEIDENBAUER

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## **ABSTRACT**

### **MUCH ADO ABOUT EATING: DIETARY THERAPY FOR HEALTH AND DISEASE MANAGEMENT**

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Dietary therapy has been used since ancient times to treat the symptoms of disease and disorder. Dietary therapy has long captured the interest of the public in modern times, dating back to the mid-nineteenth century with Englishman William Banting's "Letter on Corpulence, Addressed to the Public", which addressed Banting's anecdotal use of a high-fat diet to treat obesity. High-fat diets became popular in the United States in the early twentieth-century to treat epilepsy. The utility of dietary therapy to treat diseases and disorder has not been embraced widely, as there is a paucity of standardized clinical trials that demonstrate robust safety and therapeutic efficacy for specific diseases and disorders. Additionally, preclinical studies of dietary therapy do not adhere to standardized guidelines, which can hinder cross-study interpretation and reproducibility. To that end, my dissertation updates diet implementation guidelines for preclinical studies that adhere to standardized experimental design and biomarker monitoring in mouse models in order to maximize therapeutic efficacy, diet regimen safety, and cross-study interpretability. With these guidelines, I explored the effect of various diets on circulating glucose and ketone bodies in mice, a measure of glycolytic flux, along with biomarkers of health. I found that calorie-restricted diets, regardless of



macronutrient composition, lowers circulating glucose and increases circulating ketone levels, along with improving biomarkers of health, including lowering circulating triglyceride levels. In demonstrating the utility of dietary therapy to treat disease, I also explored the mechanisms on how dietary therapy can be used to treat epilepsy in a preclinical mouse model. I showed that reduced glucose utilization underlies the seizure-protective effects of dietary therapy in EL mice, a mouse model of idiopathic epilepsy. Lastly, I developed a novel tool, the Glucose Ketone Index Calculator, to track the progress of dietary therapy in brain cancer patients through a ratio of circulating glucose to circulating ketone bodies. Evidence is presented that demonstrates a low ratio of glucose to ketone bodies is associated with improved prognosis of brain cancer management in humans and mice. Overall, this dissertation demonstrates the utility of dietary therapy in treating disease using standardized guidelines, and suggests the use of a novel tool to apply and track the progress of dietary therapy in the clinical brain cancer population.

## DEDICATION

To my family and especially my loving parents, for always supporting me in my endeavors, and encouraging me to challenge myself.

To the Pearlstein family, thank you for always offering a kind hand in life and a warm meal when it was needed.

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## ABBREVIATIONS

2-DG	2-deoxy-D-glucose
AcAc	acetoacetate
AED	Antiepileptic drug
AL	<i>ad libitum</i>
bOHB	beta-hydroxybutyrate
C	Chloroform
CB	Cerebrosides
CE	Cholesteryl ester
Cer	Ceramide
Chol	Cholesterol
CR	Calorie restriction
FFA	Free fatty acids
FO	Fish-oil supplemented diet
FO-R	Fish-oil supplemented diet, calorie restricted
FO-UR	Fish-oil supplemented diet fed <i>ad libitum</i>
GABA	$\gamma$ -aminobutyric acid
GAD	Glutamate decarboxylase
GKI	Glucose Ketone Index
GKIC	Glucose Ketone Index Calculator
Glu	Glucose
GLUT-1	Glucose transporter type 1
HPTLC	High performance thin layer chromatography
IS	Internal standard
KB	ketone body
KD	Ketogenic diet
KD-R	Ketogenic diet, calorie restricted

KD-UR	Ketogenic diet fed <i>ad libitum</i>
LPC	Lyso-phosphatidylcholine
M	Methanol
NAA	<i>N</i> -acetylaspartate
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PCOS	Polycystic ovary syndrome
PDH	Phosphofructokinase
PFK	Pyruvate dehydrogenase
R	Restricted feeding
ROS	Reactive oxygen species
SD	Standard rodent chow diet
SD-R	Standard rodent chow diet, calorie restricted
SD-UR	Standard rodent chow diet fed <i>ad libitum</i>
SEM	Standard error of the mean
SM	Sphingomyelin
TG	Triglycerides
UR	Unrestricted feeding

## CHAPTER ONE

### INTRODUCTION

#### Dietary Therapy

Dietary therapy is defined as any change in nutrition that is intended to treat an illness, injury, or condition (Council on Practice (COP) Quality Management Committee, 1994; Pastors et al., 2002). Some of the earliest recordings of dietary therapy include the first-century Roman Celsus's writings on the use of fasting to treat ocular infection and inflammation (Celsus & Spencer, 1935; Eadie & Bladin, 2001). In the mid-nineteenth century, Englishman William Banting published his booklet on his personal experience treating his obesity with a high-fat diet, titled "Letter on Corpulence, Addressed to the Public" (Banting, 1864). In the early twentieth century, fasting was recognized as an effective therapy for epilepsy (Freeman et al., 1994; Geyelin, 1921; Lennox & Cobb, 1928; Wheless, 2008). Around the same time, the ketogenic diet was developed as a long-term alternative therapy for fasting (Wheless, 2008; Wilder, 1921). Dr. Wilder of the Mayo Clinic coined the term "ketogenic diet" to describe a diet that results in ketonemia– the presence of ketone bodies in blood (Wilder, 1921). The ketogenic diet was formulated to give children unlimited dietary fat, 1 g of protein per kg of bodyweight, and 10-15 g of carbohydrates per day (Peterman, 1925). With the advent of phenytoin in 1938, and phenytoin and phenobarbital derivatives in the 1940's and 1950's, dietary therapy

for epilepsy was largely abandoned due to the ease of compliance of oral drug delivery (Porter et al., 1984). While dietary therapy was utilized throughout much of the twentieth century to treat a variety of ailments, including diabetes, hyperlipidemia, and irritable bowel syndrome, dietary therapy was generally not viewed as a first-line therapy for most ailments (Chima, 2007; Mayo Clinic Committee on Dietetics., 1949). In the 1990's the ketogenic diet received a resurgence of interest due to the successful treatment of patients with epilepsy at John Hopkins, along with the production of the TV-movie "First Do No Harm" featuring Meryl Streep that exposed the potential of the ketogenic diet to treat epilepsy (Freeman et al., 1994; Wheless, 2008). In recent years, the interest in utilizing dietary therapy to treat widespread diseases and disorders, such as cancer and autism, has increased as case reports and anecdotal evidence suggest that dietary intervention may cure or alleviate symptoms of disease in humans (Evangelidou et al., 2003; Nebeling et al., 1995; Wilson, 2013; Zuccoli et al., 2010).

Dietary therapy, and specifically the ketogenic diet, may treat and prevent diseases through a myriad of mechanisms, including reducing blood glucose, increasing mitochondria biogenesis, reducing insulin levels, reducing IGF-1, and influencing neurotransmitter activity, to name a few (**Figure 1**) (Paoli et al., 2013; Paoli et al., 2014). Diseases that can potentially be managed by dietary therapy include epilepsy, Alzheimer's disease, Parkinson's disease, traumatic brain injury, migraine, diabetes, obesity, autism, and cancer (**Table 1**) (Baranano & Hartman, 2008; Neal, 2014; Stafstrom & Rho, 2012). The acceptance of dietary therapy as an

appropriate treatment modality is contingent on rigorous analyses that demonstrate robust therapeutic efficacy. Due to the preponderance of pharmacological compounds as a first-line treatment for a variety of diseases and disorders, preclinical studies utilizing dietary therapy are often needed to demonstrate safety and therapeutic efficacy before human trials are conceived and conducted. Therefore, standardized guidelines are needed to increase preclinical study validity and reproducibility with respect to dietary therapy as a primary and complementary therapeutic modality. Accordingly, chapter two of this dissertation updates diet implementation guidelines for preclinical studies that adhere to standardized experimental design and biomarker monitoring in mouse models in order to maximize diet regimen safety, therapeutic efficacy, and cross-study data interpretability.

### Glucose and Ketone Bodies

While there are many forms of dietary therapy, this dissertation focuses specifically on dietary therapies that are intended to shift cellular energy metabolism from glucose- and lactate-based metabolism to ketone-based metabolism. Under normal conditions, cells preferentially use glucose (or lactate) as an energy substrate (Magistretti & Pellerin, 1999; Magistretti & Allaman, 2013). Sources of glucose include dietary carbohydrates, glycerol, and glucogenic amino acids, along with stored glycogen and glucogenic amino acids.

Under fasted conditions and calorie-restricted conditions, many cells in the human body can utilize ketone bodies as an energy substrate, including brain, heart, and muscle cells (Owen et al., 1967). The water-soluble ketone bodies beta-hydroxybutyrate (bOHB) and acetoacetate (AcAc) are metabolizable energy substrates that provide energy in the absence of glucose, whereas acetone is a volatile ketone body byproduct of AcAc decarboxylation that is excreted through the lungs (Han et al., 2011).  $\beta$ -hydroxybutyrate composes approximately 80% of circulating ketone bodies, AcAc accounts for approximately 20% of circulating ketone bodies, and acetone accounts for <1% of circulating ketone bodies (Cahill & Veech, 2003).  $\beta$ -hydroxybutyrate and AcAc are readily interconverted in the liver to maintain a 4:1 bOHB:AcAc circulating equilibrium (Hashim & VanItallie, 2014; Veech, 2004; Williamson et al., 1962). Under prolonged fasting, the human brain can derive over half of its energy from ketone body metabolism (**Table 2**) (Cahill, 2006; Hashim & VanItallie, 2014; Morris, 2005; Owen et al., 1967; Veech, 2004). Sources of ketone bodies for ketogenesis and ketone metabolism include dietary fatty acids and ketogenic amino acids, along with stored fatty acids and ketogenic amino acids. Ketogenesis occurs primarily in the liver. There is evidence that the renal medulla is capable of ketogenesis during periods of hepatic ischemia (Nakatani et al., 1999; Weidemann & Krebs, 1969; Zhang et al., 2011). Astrocytes in culture are also capable of ketogenesis (Auestad et al., 1991; Blazquez et al., 1999; Guzman & Blazquez, 2001; Guzman & Blazquez, 2004).



Recent research has uncovered broad neuroprotective properties of ketone bodies. Acetoacetate exerts anticonvulsant effects in Frings audiogenic-susceptible mice (Rho et al., 2002).  $\beta$ -hydroxybutyrate protects against dopaminergic cell loss in a mouse model of Parkinson's disease and in cultured neurons (Imamura et al., 2006; Tieu et al., 2003).  $\beta$ -hydroxybutyrate also protects cultured hippocampal neurons against amyloid-beta toxicity (Kashiwaya et al., 2000). Ketone bodies have improved neuronal function in mouse models of hypoxia and ischemia (Suzuki et al., 2001). Additionally, ketone bodies have garnered much interest for their potential to ameliorate symptoms of traumatic brain injury (Davis et al., 2008; Prins & Matsumoto, 2014). Ketone bodies are believed to mediate their neuroprotective effects through enhancing mitochondrial respiration, increasing ATP production, reducing reactive oxygen species (ROS), and enhancing anti-apoptotic signaling through reducing cytochrome c release (**Figure 2**) (Hu et al., 2009; Maalouf et al., 2007; Maalouf & Rho, 2008; Maalouf et al., 2009; Suzuki et al., 2001; Veech, 2004).

The neuroprotective effects attributed to ketogenic diets are likely mediated partly through ketone bodies (**Figure 1, Table 1**). Additionally, the same bioenergetic state required to produce ketone bodies, decreased energy availability, also increases intracellular and extracellular adenosine, which is an inhibitory purine that can reduce excitotoxicity (Dunwiddie & Masino, 2001; Masino & Geiger, 2008). The ketogenic diet is also posited to exert its neuroprotective effects through reducing glycolytic flux (Stafstrom & Rho, 2012), although the ketogenic diet needs to be calorie restricted to observe these effects (Meidenbauer et al., 2014).

In order to study dietary therapy in the context of disease management, it is necessary to understand how dietary changes affect glycolytic flux and ketogenesis. Accordingly, chapter three of this dissertation examines the effects of dietary therapy on circulating glucose and ketone bodies and other biomarkers of health. Diets were analyzed under both *ad libitum* and calorie-restricted conditions to parse the effects of macronutrient composition from caloric consumption.

### Dietary Therapy in Disease Management: Epilepsy

Epilepsy is a chronic neurological disorder that results from aberrant neuronal paroxysmal discharges that produce seizures (Engel, 1997). Over 50 million people worldwide are affected by epilepsy (Banerjee et al., 2009). Epileptic seizures are classified as either generalized or focal (Berg et al., 2010). Generalized seizures originate at some point in the brain and generalize throughout both hemispheres, whereas focal seizures originate within one hemisphere of the brain and do not bilateralize to the contralateral hemisphere. Generalized seizures can be further classified as tonic-clonic, absence, myoclonic, clonic, tonic, and atonic seizures. Approximately two-thirds of individuals with epilepsy have their seizures managed through antiepileptic drug (AED) treatment (Kwan & Brodie, 2000). The first drug treatment in modern times for epilepsy was potassium bromide in the mid-nineteenth century, followed by barbiturates in the 1930's and 1940's. Present-day AED treatment of epilepsy consists of a multitude of drug classes to treat specific seizure disorders. Benzodiazepines are prescribed at a high rate, and treat a

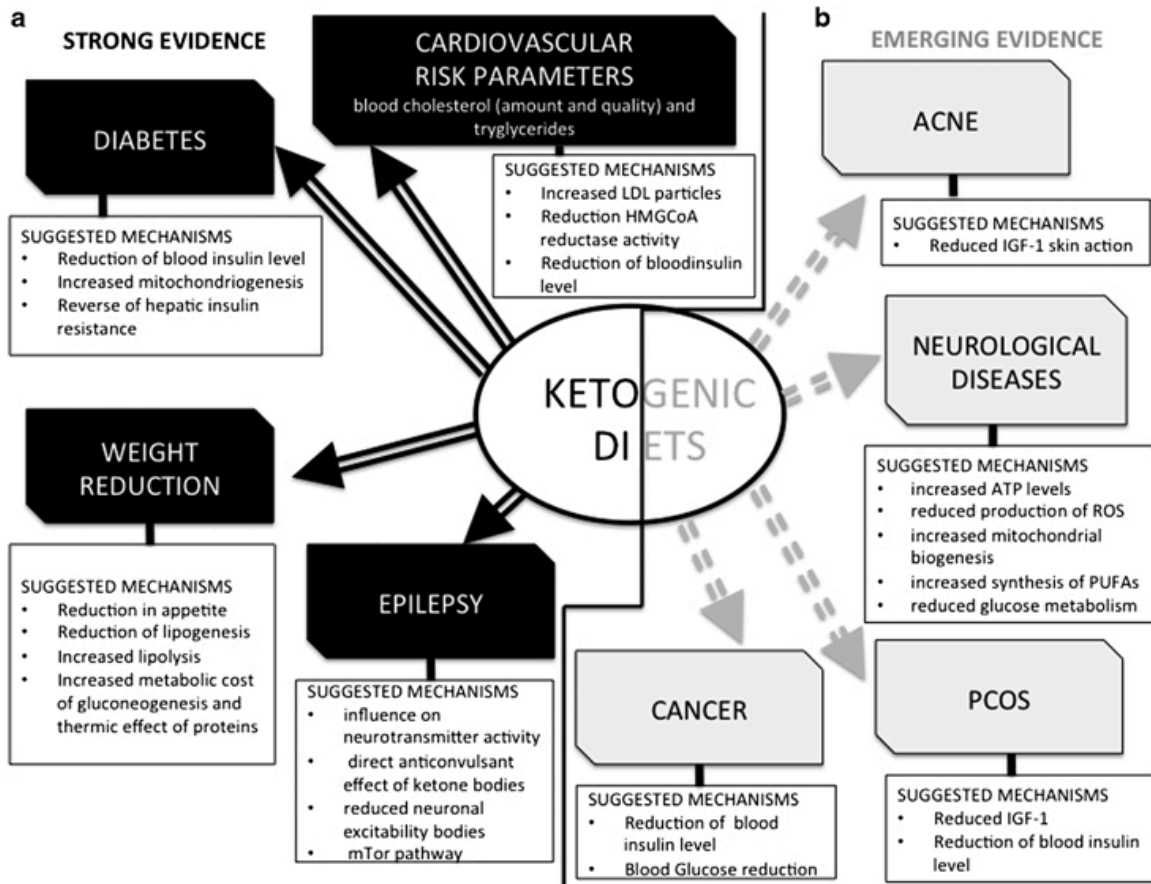
broad class of seizure disorders (Riss et al., 2008). For the approximately one-third of patients that cannot manage seizures with AED therapy alone, the ketogenic diet is increasingly prescribed as an alternative treatment, although the rates of diet utilization remain low (Lutas & Yellen, 2013). The ketogenic diet is able to provide seizure management for some individuals with drug-resistant epilepsy. For individuals that receive the ketogenic diet as treatment for epilepsy, approximately 38% experience a 50% or greater reduction in seizure frequency, compared to controls (Levy et al., 2012). The mechanisms for how the ketogenic diet, and other dietary therapies more generally, protect against seizures in epilepsy are not well understood. Chapter four examines the mechanisms through which dietary therapy confers seizure protection in a mouse model of epilepsy.

#### Challenges of Studying and Utilizing Dietary Therapy for Disease Management in the Clinic

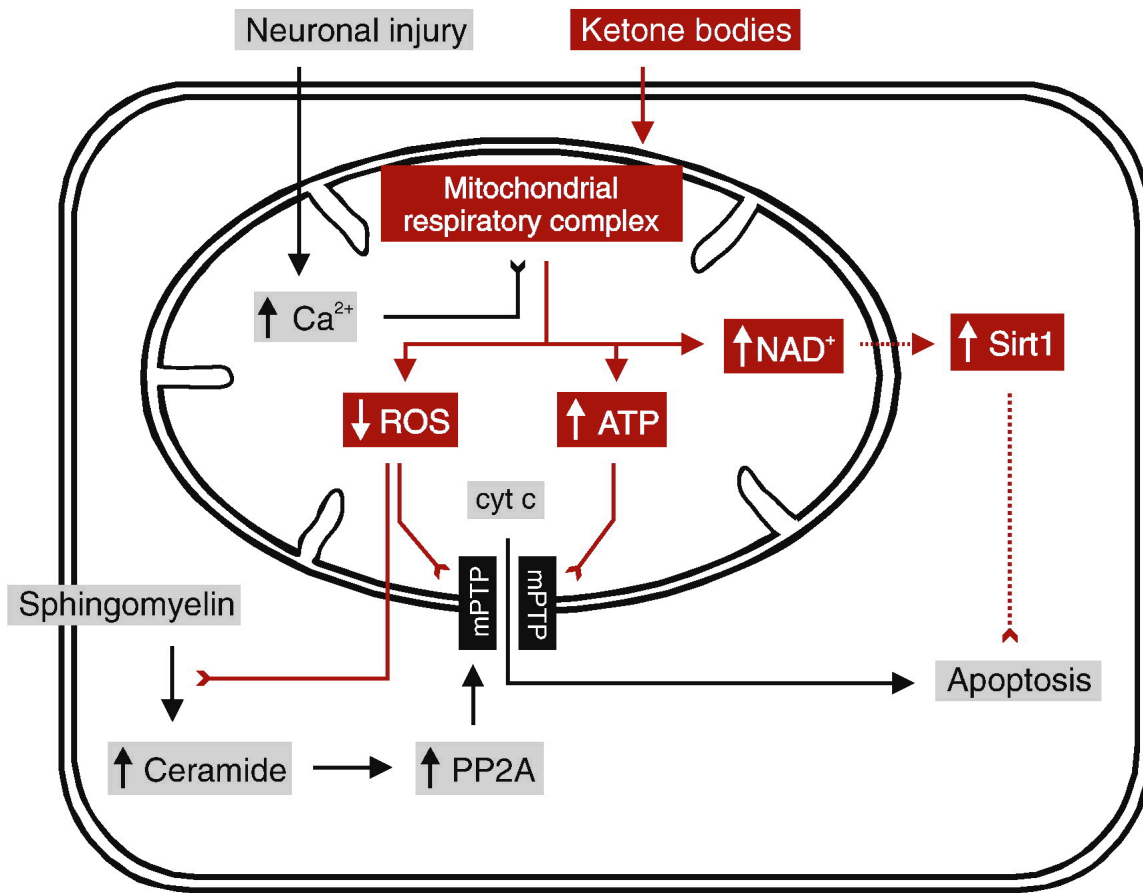
Implementing and tracking the progress of dietary therapy in human disease management remains difficult and unstandardized. Due to the lack of standardization, it is difficult to fully evaluate the therapeutic efficacy and safety of dietary therapy through disease- and treatment-specific meta-analyses. Defining clinical biomarkers that are predictive of disease management can facilitate the implementation and tracking of dietary therapy. To that end, chapter five of this dissertation explores the development of a clinical biomarker and a tool to assess the clinical biomarker for brain cancer management with dietary therapy.

My dissertation research updates guidelines for implementing dietary therapy in mouse models of disease and disorders. This dissertation also examines how various diets affect circulating glucose and ketone bodies in mice, a measure of glycolytic flux. Importantly, my research shows that calorie-restricting diets, regardless of macronutrient composition, lowers circulating glucose and increases circulating ketone bodies, along with improving biomarkers of health (Meidenbauer et al., 2014). My work goes on to further understand how dietary therapy confers seizure protection in the EL mouse, a mouse model of idiopathic epilepsy. I show that reduced glucose utilization underlies seizure protection with dietary therapy in EL mice (Meidenbauer & Roberts, 2014). Finally, I developed a novel tool, the Glucose Ketone Index Calculator (GKIC), to track the ratio of circulating glucose to ketone bodies, a useful clinical biomarker for assessing the progress of dietary therapy in disease management. The GKIC is validated through analyzing brain tumor management with dietary therapy in both preclinical and clinical studies.

**Figure 1.** Diseases managed by the ketogenic diet and evidence-based mechanisms. Cardiovascular disease, diabetes, obesity, and epilepsy have **(a)** strong evidence for management, while other neurological disorders, cancer, acne, and Polycystic Ovary Syndrome (PCOS) have **(b)** emerging evidence for management. Figure reproduced with permission from Paoli, Rubini, Volek, & Grimaldi. (2014). *European journal of clinical nutrition*, 68, 641.



**Figure 2.** Neuroprotective mechanisms of ketone bodies. Ketone bodies mediate neuroprotective effects through increasing complex-I driven mitochondrial respiratory function, increasing ATP synthesis, decreasing ROS, and increasing anti-apoptotic signaling. The effects of neuronal injury are highlighted in gray, and the effects of ketone bodies are highlighted in red. Figure reproduced with permission from Maalouf, Rho, & Mattson. (2009). *Brain Research Reviews*. 59(2), 293-315.





**Table 1. Potential uses of dietary therapy in various illnesses (clinical and laboratory studies)<sup>1</sup>**

<b>Disorder</b>	<b>Potential mechanisms of action</b>
<b>Neurologic</b>	
Epilepsy	Enhanced mitochondrial function; fatty acid effect on ion channels, neurotransmission and neurotransmitters; decreased effects from reactive oxygen species; reduced glucose utilization
Alzheimer disease / cognitive impairment	Decreased beta amyloid deposition
Parkinson's disease	Enhanced mitochondrial function
Amyotrophic lateral sclerosis	Enhanced mitochondrial function
Traumatic brain injury	Ketone body utilization, enhanced mitochondrial function
Hypoxic / ischemic brain injury	Ketone body utilization, enhanced mitochondrial function
Autism	Enhanced mitochondrial function
Depression	Enhanced mitochondrial function
Migraine	Enhanced mitochondrial function
Narcolepsy	Enhanced mitochondrial function
<b>Metabolic</b>	
Cancers expressing the Warburg effect	Ketone body utilization, enhanced mitochondrial function
GLUT-1 deficiency <sup>2</sup>	Ketone body utilization
PDH deficiency <sup>3</sup>	Ketone body utilization
PFK deficiency <sup>4</sup>	Ketone body utilization
McArdle disease (Glycogenosis type V)	Ketone body utilization
<b>Other</b>	
Cardiac ischemia	Ketone body utilization, enhanced mitochondrial function

<sup>1</sup>Modified from Baranano & Hartman. (2008). *Current treatment options in neurology*. 10(6), 410-419.<sup>2</sup>GLUT-1, glucose transporter type 1<sup>3</sup>PDH, pyruvate dehydrogenase<sup>4</sup>PFK, phosphofructokinase

**Table 2. Proportion of brain energy metabolism supported by ketone bodies, as a function of plasma ketone body (KB) concentration.<sup>1</sup>**

<b>Plasma KB concentration (mM)</b>	<b>Fasting time to achieve plasma KB concentrations</b>	<b>Proportion of brain energy metabolism</b>
0.3 - 0.5	12-24 hrs	3 - 5%
1.5	2-3 days	18%
5	8 days	60%
7 - 8	≥ 20 days	> 60%

<sup>1</sup>Modified from Hashim & VanItallie. (2014). *Journal of Lipid Research*. 55(9), 1818-1826.

## CHAPTER TWO

### *Guidelines for the implementation of dietary therapy in mice to optimize translational research*

#### INTRODUCTION

Dietary therapies can be effective in delaying the onset or in mitigating the adverse consequences of neurological and non-neurological diseases and disorders, as evidenced by numerous preclinical and clinical studies conducted over the past century (Estruch et al., 2013; Gasior et al., 2006a; Hursting et al., 2003; Neal et al., 2008; Peterman, 1925; Sacks & Katan, 2002; Yancy et al., 2004). Dietary therapy is prescribed as either a change in macronutrient content in an *ad libitum*-fed diet, or by restricting calorie intake. Examples of diets that utilize macronutrient content changes include the Mediterranean diet, the ketogenic diet, and gluten- and casein-free diets (Elder et al., 2006; Sharman et al., 2002; Willett et al., 1995). Calorie restriction (CR) can be implemented as a primary therapy or as a complementary to a macronutrient-specific diet (Mantis et al., 2004; Meidenbauer et al., 2014). CR can be achieved through a variety of protocols, including lowered daily calorie intake or intermittent fasting (Moreira et al., 2011; Varady et al., 2009). Successful dietary therapy implementation utilizes one or more of these protocols to achieve the desired therapeutic effect.

The present guidelines describe the methods that I have refined and utilized to implement dietary therapy in mice. To my knowledge, the last comprehensive guidelines for dietary implementation in mice, which focused specifically on calorie restriction, were described 15 years ago (Pugh et al., 1999). It is important to continually update guidelines through evidence-based formation and to make these available to the research community. A brief survey of the literature is presented to demonstrate evidence-based formation of these guidelines. Many methods, however, have also been optimized through personal experience. These guidelines are not all encompassing, as each study design presents unique challenges. I will discuss the specific cases of implementing dietary therapy in the form of CR and the ketogenic diet (KD), but many of the principles presented can be utilized for implementing other dietary modalities.

#### Considerations for Preclinical-to-Clinical Translation

It is important to consider the numerous physiological differences between mice and humans that can confound data interpretation and extrapolation, with respect to the effect that diet has on disease progression and outcome. Specific oxygen consumption rate, which is a measure of aerobic energy metabolism per unit mass, is approximately eight-fold greater in mice than in humans (Davies & Morris, 1993). When total metabolism is considered, mice are oft-quoted as having a seven-fold greater specific metabolic rate, which is generated using Max Kleiber's power

function of body weight (Kleiber, 1947). This difference becomes important as the effects of diets and fasting on mice and humans are considered.

The increased specific metabolic rate in mice compared to humans has important consequences on translating nutrient intake from mice to humans. Considering a 70 kg human consumes ~2,000-2,500 kilocalories per day, and a 25 g mouse consumes ~10-15 kilocalories per day, specific calorie consumption per day is approximately 15-20 fold greater in mice than in humans. This difference means that dietary manipulation will have greater short-term effects on a mouse than on a human (Terpstra, 2001). Therefore, caution is required in extrapolating positive or negative dietary effects from mice to humans.

Due to the increased metabolic rate of mice, shorter fasting periods of 5-6 hours are sufficient to mimic an overnight fast (14-18 hours) in humans (Ayala et al., 2010). Fasting periods as short as 1-3 hours can be used to obtain baseline blood biomarker values in mice (Mantis et al., 2004). Overnight fasting in mice (18 hours) leads to a catabolic state and enhances insulin-stimulated glucose utilization, whereas humans have impaired insulin-stimulated glucose utilization after prolonged fasting (Ayala et al., 2006; Ayala et al., 2010). The response to overnight fasting in mice needs to be considered when measuring metabolites or hormones that may change significantly with prolonged fasting.

Because the therapeutic efficacy of dietary therapy can be related to changes in specific biomarkers, such as glucose and ketones (Greene et al., 2001; Mantis et al., 2004; Mantis et al., 2014), it is important to identify the desired biomarker

changes prior to initiating a dietary therapy. Any dietary therapy that is meant to change metabolic homeostasis should include measurements of blood glucose and ketone bodies ( $\beta$ -hydroxybutyrate), as the relative amounts of these metabolites can be indicative of carbohydrate or fat metabolism, respectively (Ding et al., 2013; Greene et al., 2003; Seyfried et al., 2003; Yao et al., 2011).

Blood is an excellent tissue for monitoring shifts in biomarkers of metabolic homeostasis, as long as blood collection methods (specifically volume and frequency of collection) do not alter the metabolic state. A more thorough treatment on specific blood collection methods and how metabolite levels can be changed with repeated sampling is discussed elsewhere (Durschlag et al., 1996; Parasuraman et al., 2010; Tabata et al., 1998). It is important to note that blood should be collected using the same procedure for the duration of the study, as changing the method of collection can change the value of the biomarkers being monitored (Fernandez et al., 2010; Heimann et al., 2010). This becomes important at the end of a study, as investigators have reported significant differences in biomarker values between non-terminal and terminal blood collection procedures (Vaquero et al., 2011).

### Implementing Novel Diets

It is necessary to determine the tolerability of a novel diet on mice before initiating the dietary therapy. With well-tolerated diets, mice maintain body weight (under *ad libitum* feeding conditions) without displaying signs of lethargy, malnutrition or dehydration (Foltz & Ullman-Cullere, 1999; Ullman-Cullere & Foltz,

1999). Calorie restricted diets are considered well-tolerated when the mouse's prescribed body weight loss can be achieved and maintained for the duration of the study, along with the absence of signs of malnutrition or dehydration.

Laboratory mice may self-restrict food intake when a new diet is introduced (personal observations). Self-restriction of food intake can be age- and strain-specific. Juvenile mice (30-60 days of age) are more likely to accept a new diet than are older conspecifics. A temporary water-only fast for 12-24 hours can be used to prevent self-restriction before initiating a new diet. A period of 12 hours of fasting is sufficient for juvenile mice, whereas adult mice can be fasted for up to 24 hours before implementing the new diet (Denny et al., 2006; Denny et al., 2010; Mahoney et al., 2006; Mantis et al., 2004; Mantis et al., 2009; Marsh et al., 2008a; Marsh et al., 2008b; Seyfried et al., 2003; Zhou et al., 2007). The Seyfried laboratory has found that 8 hours of water-only fasting is adequate for juvenile mice and 12-16 hours of fasting for adult mice (Greene et al., 2001; Mantis et al., 2004). A temporary fast will bring all animals to a similar metabolic set point before initiation of a new diet (Reed et al., 1991).

#### Implementing Dietary Therapy – Single- or Group-housed mice?

It is important to determine whether single- or group-housing of mice is most appropriate for the study outcomes. Single housing allows precision in tracking food intake, and allows the use of metabolic chambers to precisely quantify an individual mouse's metabolism. Conversely, it is important to recognize that

social isolation introduces anxiety-related stress that can confound study results in rodents (Matsumoto et al., 2005; Wu et al., 2000). A lack of social contact can influence the behavior of mice, especially in social-oriented behavioral tasks (Bartolomucci et al., 2003; Palanza et al., 2001). Single housing can increase behavioral arousal to novel stimuli and can induce social stress (Matsumoto et al., 2005; Wu et al., 2000). Additionally, exposure to a novel environment (such as a behavioral testing apparatus) can increase basal corticosterone levels and alter metabolic homeostasis more in singly housed mice than in group housed mice (Bartolomucci et al., 2003). Mice that are single-housed require a higher ambient temperature than group housed mice to maintain body temperature and minimize metabolic and thermoregulatory stress (Gordon et al., 1998). Placing cotton-nesting pads into the cages of mice that are single-housed or increasing the temperature of the room can achieve this. Deviations from normal body temperature in mice have been shown to alter food and water intake, dependent on strain (Yamauchi et al., 1983). Additionally, mice that are single-housed after neurological injury or surgery have demonstrated impaired recovery, compared to group-housed mice. Impaired recovery can be minimized by providing an enriched cage environment to single-housed mice (Will et al., 2004).

Group housing is an alternative to single cage housing. An obvious drawback to group housing is that food intake cannot be accurately measured for individual mice in the group. Instead, the food intake can be measured for the group and then estimated as the mean intake for the individuals in the group. Group housing of mice



allows social contact, which is important for studies that assess behavior. Hence, studies of behavior and social function may necessitate group housing of mice.

While group housing is optimal for dietary studies where behavioral data are the primary endpoints, the administration of a specific diet under these conditions can be challenging, especially for studies involving calorie restriction. Before beginning a dietary study with group housed mice, it is important to consider the strain and sex of the mice that will be used. Implementing dietary therapies using group-housed mice from more aggressive strains can be difficult, as a social hierarchy may be quickly established in the cage (Avitsur et al., 2001; Long, 1972; Louch & Higginbotham, 1967; Schuhr, 1987). This can lead to issues such as uneven weight loss/gain, social stress, and metabolic/physiological changes. The sex of mice is also an important consideration, as female mice are generally more docile than male mice (dependent on reproductive status). It has been our experience that female mice and mice of docile strains respond more favorably to dietary implementation in group housed settings than male mice and mice of aggressive strains. For information on the general aggressiveness of a variety of inbred mouse strains, consult the review by Crawley *et al.* (Crawley et al., 1997), along with other published literature (Brodkin et al., 2004; Guillot et al., 1994; Guillot & Chapouthier, 1996; Jones & Brain, 1987; Miczek et al., 2001).

Mice that are group housed should be closely matched for body weight. Mice that differ greatly in body weight (> 10%) can have very different caloric intake requirements (Pugh et al., 1999). This can lead to disproportionate weight gain in

some mice and weight loss in other mice. Caloric intake requirements can differ even in mice with similar body weights (Mantis et al., 2004; Meidenbauer et al., 2014). Feeding paradigms for group housed mice should be tested among the specific strain and sex of mice that will be used for a study. Mice can receive their food from the cage hopper under *ad libitum*-fed dietary conditions. On the other hand, alternative-feeding paradigms may be needed for mice given a dietary intervention. I have used cage dividers during the first hour of feeding for group-housed mice under calorie restriction (**Figure 3**). I find that mice under calorie restriction will eat most of their food within the first hour of daily food administration (personal observations). As a result, a cage divider is generally needed only for the first hour after food administration. After the cage divider is removed, social contact among the mice will resume and all mice will have free access to the remaining, unconsumed food. Using this method, I have had success in implementing calorie restriction among juvenile mice in a group-housed setting (**Figure 4**).

#### Calorie Restriction – Important Considerations

Although a variety of forms of CR have been implemented with different levels of feeding restriction, CR is typically accomplished by an approximate 15-50% decrease in total calorie intake (Koubova & Guarente, 2003; Mantis et al., 2004; Masoro, 2009; Pugh et al., 1999). The most widely-used method for implementing CR is pair-feeding, whereby CR mice are restricted to 30-50% of daily food intake of

an unrestricted group (Feige et al., 2008). For calorie-restricted diets, some investigators supplement vitamins and minerals to match micronutrient intake of *ad libitum*-fed mice (Birt et al., 1991; Pugh et al., 1999; Weindruch et al., 1988). Other investigators have successfully implemented long-term calorie-restricted diets without supplementing vitamins and minerals (Goodrick et al., 1982; Keenan et al., 1996).

The use of each mouse as its own body weight control is an alternative to the pair-feeding procedure for implementing CR in mice (Mantis et al., 2004; Seyfried et al., 2008; Zhou et al., 2007). Using each mouse as its own body weight control reduces variability in body weights among mice that are fed diets differing in nutritional composition and caloric content (Meidenbauer et al., 2014; Seyfried, 2012) (**Figure 5**). It is also more accurate to use body weight as an index for determining the percentage of CR (Seyfried et al., 2003; Zhou et al., 2007). This yields more reliable measurements of blood metabolites, disease incidence and progression, and behavioral effects following CR (Mantis et al., 2004; Mantis et al., 2009; Marsh et al., 2008b). This can also counteract the swings of bodyweight due to the phenomenon of hyperphagia (binge eating) resulting from cerebral hyperglycolysis, which can be observed in mice with acute cerebral injury (e.g. cerebral tumor implantation) (Mukherjee et al., 2002; Seyfried et al., 2003). In addition, establishing metabolic (e.g. body weight, food intake, glucose, ketones) and disease phenotypes (e.g. seizure frequency, behavior, tumor growth etc.) before dietary treatment for each animal further enables reliable implementation of CR and

its effects on measurable outcomes (Greene et al., 2001; Mantis et al., 2004; Mantis et al., 2009). It should also be recognized that CR in a mouse could simulate intermittent fasting in humans. Mice on CR will often consume all of their food immediately after administration (within 1-4 hours). The mouse would then go without food for about 20-23 hours. This fast time in the mouse would be comparable to a multi-day fast in humans.

The restriction of calories in juvenile mice requires care to avoid malnutrition. A transition from suckling to the consumption of a CR diet can induce malnourishment in some mice resulting in body weight loss and impaired brain development (Thavendiranathan et al., 2003). It is imperative to ensure that no developmental delays occur in young mice on a CR diet that might impact behavior, disease characteristics, or metabolism at later ages. The growth rate of juvenile mice on CR should be similar to that of juvenile on an *ad libitum*-fed diet (Greene et al., 2001; Todorova et al., 2000) (**Figure 4**). It is vital that the percent body weight reduction for young mice fed a restricted diet is not greater than 8-15% in order to avoid malnutrition. In contrast to young mice, older cohorts can tolerate a greater degree of CR or body weight reduction without developing symptoms of malnutrition (Denny et al., 2006; Mahoney et al., 2006; Mantis et al., 2009). A 30-65% reduction in calorie intake is well-tolerated among most adult mice (> 90 days of age), and should produce a 15-25% reduction in body weight (Mantis et al., 2004).

Active body weight controls are necessary for treated animal groups that experience any degree of body weight loss from the treatment. The active body weight control group is an untreated group that is calorie restricted to match the percentage reduction in body weight of that in the treated group. This is done to rule out confounding variables related to inadvertent CR. For example, if a specific macronutrient diet is proposed to reduce disease or improve health outcomes, it is necessary to address the extent to which the improvement is due specifically to the treatment or to secondary effects of body weight reduction from CR (Morley et al., 2010; Mukherjee et al., 2004; Seyfried, 2012).

### Ketogenic Diets

Ketogenic diets are high in fat and low in carbohydrates and protein. A ketogenic diet can be further defined by its ketogenic ratio, which is a ratio of grams of fat to grams of protein plus carbohydrates in the diet (Wirrell, 2008). Implementing dietary therapy with ketogenic diets follows the principals outlined in the section 'Implementing Novel Diets'. Mice introduced to some ketogenic diet formulations may self-restrict food intake and lose bodyweight (Kennedy et al., 2007). Mice fasted overnight, prior to the introduction of the ketogenic diet, may be less likely to self-restrict on the diet (Meidenbauer et al., 2014). If mice do lose bodyweight while on a ketogenic diet, it is important to use active body weight controls to parse the effects of the calorie restriction from the high-fat feeding. It is also important to replace the ketogenic food daily in mouse cages, as the ketogenic

diet is high in fat and will become rancid. I have observed that some ketogenic diets develop significant rancidity at room temperature after 24 hours. Rancidity can independently reduce food intake leading to inadvertent body weight loss. It is recommended that the food should be stored at 4°C and be prepared fresh daily.

#### Combining Pharmacological Therapy and Dietary Therapy – Avoiding Pitfalls

It may be necessary to combine pharmacological agents with dietary therapy to test for additive and synergistic effects against disease and disorders. It is necessary to first determine the effects that a specific pharmacological compound may have on dietary therapy. The route of administration and the compound's properties may affect calorie intake and body weight. If body weight or calorie intake is changed with a given route of administration and compound, it will be necessary to utilize active body weight control mice to parse the effects of body weight gain or loss. When administering compounds orally, it is important to consider how the diet as a whole is administered. For example, administering a diet with an alkaloid compound in dry form may not influence dietary intake, whereas mixing the diet and compound in water may lead to bitter tastes that reduce calorie intake (personal observations). Some compounds may need to be administered through gavage feeding. It is important to ensure that gavage-fed controls are also present in the study design, as gavage feeding can increase stress in mice (Arantes-Rodrigues et al., 2012; Balcombe et al., 2004). For a thorough treatment on the topic

of administering pharmacological compounds through diets, consult the review by Feige *et al.* (Feige et al., 2008).

## GUIDELINES TO IMPLEMENTING DIETARY THERAPY

The following guidelines describe the methods that I have refined and utilized to implement dietary therapy in mice. These specific methods, while formed through an evidence-based review of the literature, have been optimized through years of personal experience.

### Establish Baselines

It is important to establish baselines prior to initiating dietary therapy. These baselines can be used to track the effects of the dietary therapy (i.e. the secondary end-point of the study) on metabolism and behavior. Additionally, if the effect of diet on behavior is to be measured, behavioral baselines should be established prior to implementing dietary therapy. The pre-trial period needed to establish a baseline for metabolic and behavioral parameters should be about 4-10 days. Mice housing should be similar during the pre-trial period and for the duration of the study. If the mice are to be group-housed, they should be housed together with the same cage mates throughout the study. The changing of cage-mates can disrupt social exploration and increase stress levels in mice (Bartolomucci et al., 2001; Misslin et al., 1982). Mice within each experimental group should be body-

weight matched during the pre-trial period, as this will eliminate confounding effects of body weight change later in the study (Seyfried & Mukherjee, 2005a).

Mice should be maintained on a standard rodent chow diet during the pre-trial period. Body weight for each mouse, and food intake for each cage, should be recorded daily for the pretrial period. It is suggested that circulating levels of glucose and ketone body ( $\beta$ -hydroxybutyrate) be recorded during the pre-trial period, as these two metabolites can provide information on the metabolic status of the mice (Greene et al., 2001; Kennedy et al., 2007; Meidenbauer et al., 2014). A fast of 1-3 hours in mice is sufficient to obtain baseline measurement of glucose and  $\beta$ -hydroxybutyrate (Mantis et al., 2004; Zhou et al., 2007).

#### Body Weight Stabilization – Experimental Phase

Successful implementation of dietary therapy commences when the body weights of the mice have stabilized at the target body weight value (i.e. body weight reduction or maintenance). During the active experimental phase, observations can be made regarding the influence of diet on disease outcomes, and changes in specific biochemical, physiological, and behavioral biomarkers. It is important to maintain body weights at their target set point throughout this phase. A drastic change in body weight (i.e. a > 10% further body weight reduction) from the set point should signal the end of the experimental phase.



## IMPLEMENTING DIETARY THERAPY – EXAMPLES FROM START TO FINISH

### *Influence of Calorie Restriction on Brain Tumor Growth*

The response of brain tumor growth to calorie restriction can illustrate the salient points outlined in this chapter. The single housing of mice is best for these study designs, as mice from some strains could cannibalize the growing tumor from cage mates, especially under calorie-restricted conditions (personal observations). The pre-trial period can last seven days (**Figure 6**). At the start of the pre-trial period the mice are separated into individual cages, and the body weight of each mouse is measured. The mice are fasted for three hours to obtain baseline blood glucose and  $\beta$ -hydroxybutyrate levels. Food intake is measured for each mouse to establish the food intake baseline.

The tumor is implanted at the end of the pre-trial period. For the next three days, food is administered *ad libitum* (AL) for both groups to allow the tumor to grow. The mice are fasted in the evening on day three (14-16 hours) to facilitate diet initiation. After the fast, mice are weighed daily or every-other-day depending on the type of diet used. Food for the control AL group is weighed before placing it in the cage hopper. For the experimental CR group, food will be administered initially as a 40% reduction from the amount consumed during the pre-trial period. As the CR mice lose body weight, the food amount provided is adjusted to reach the targeted body weight reduction, which is a percentage of the pretrial weight of an individual mouse. The targeted body weight reduction should be reached within seven days of CR. Stable body weight should be maintained through the course of

the study for both the AL and CR mouse groups. Blood glucose and bOHB can be measured at two-week intervals or at the end of the study. Data can be collected for food intake, body weight, tumor growth and circulating levels of glucose and ketone bodies (bOHB). Examples of this approach have been previously described (Mukherjee et al., 2002; Mukherjee et al., 2004; Zhou et al., 2007).

#### *Effects of the Ketogenic Diet on Autistic-Like Behavior in Mice*

The behavioral response to the ketogenic diet in mice with autism is another example illustrating how a dietary therapy can be implemented. In this study design, group-housed mice are used to prevent complications of social isolation on the behavioral tests used. A 7-day pretrial period is initiated, as outlined in **Figure 7**. Behavioral testing is done on days 5 – 7 of the pre-trial period. Mice are fasted for 1 hour to obtain baseline blood glucose and ketone values after completion of behavioral testing. Body weights are also measured. Food intake is tracked for each cage to estimate baseline food intake during the pre-trial period. Following the pre-trial period, the mice are fasted overnight for 16 hours.

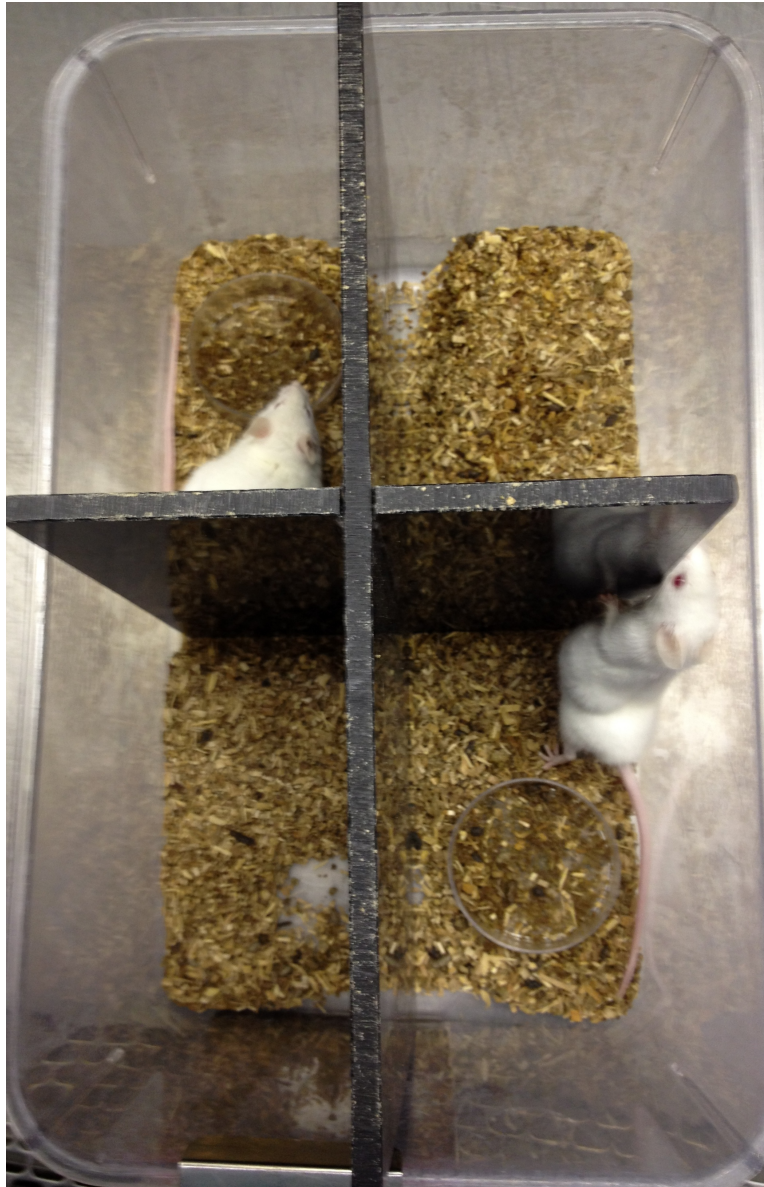
The mice are split into two groups: a control group that receives a standard mouse chow diet (SD) and an experimental group that receives a lard-based ketogenic diet (KD). Food is administered daily and the amount consumed is determined as the difference in food weight before and after a 24 hour period. The ketogenic diet is replaced daily to prevent the food from becoming rancid. The mice are fed the diets for four weeks. Body weight is measured once per week. The

behavioral tests conducted during the pretrial period are repeated on days 5, 6, and 7 of week 4. Mice are fasted as before, and blood is collected after behavioral testing.

## CONCLUSION

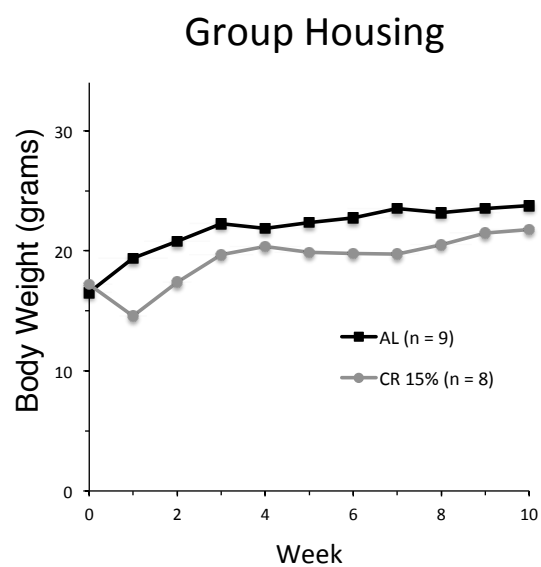
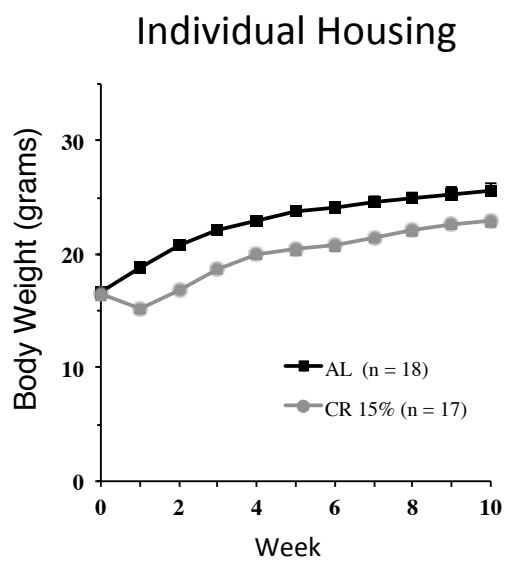
A series of guidelines are presented that can facilitate the implementation of diet therapies for mice. Successful implementation of dietary therapy is achievable through the use of a standard set of guidelines. These guidelines can help control variables to enhance the accuracy and reproducibility of results.

**Figure 3.** Cage divider for instituting calorie-restricted dietary therapy in group-housed conditions. An opaque cage divider can be used to administer food to calorie-restricted mice under group-housed conditions. Leaving the divider in for one hour is sufficient for the mice to eat most of their administered food. After one hour, the divider is removed and the remaining food is left in the cage for the mice to finish eating.



**Figure 4.** Body weight gain in juvenile EL mice under individual- and group-housed conditions. With a 15% calorie reduction instituted by week one for CR groups, body weight gain paralleled the *ad libitum* (AL) fed groups under both conditions. The 'Individual Housing' panel is modified from Greene *et al.* (2001). *Epilepsia*. 42, 1371-1378.

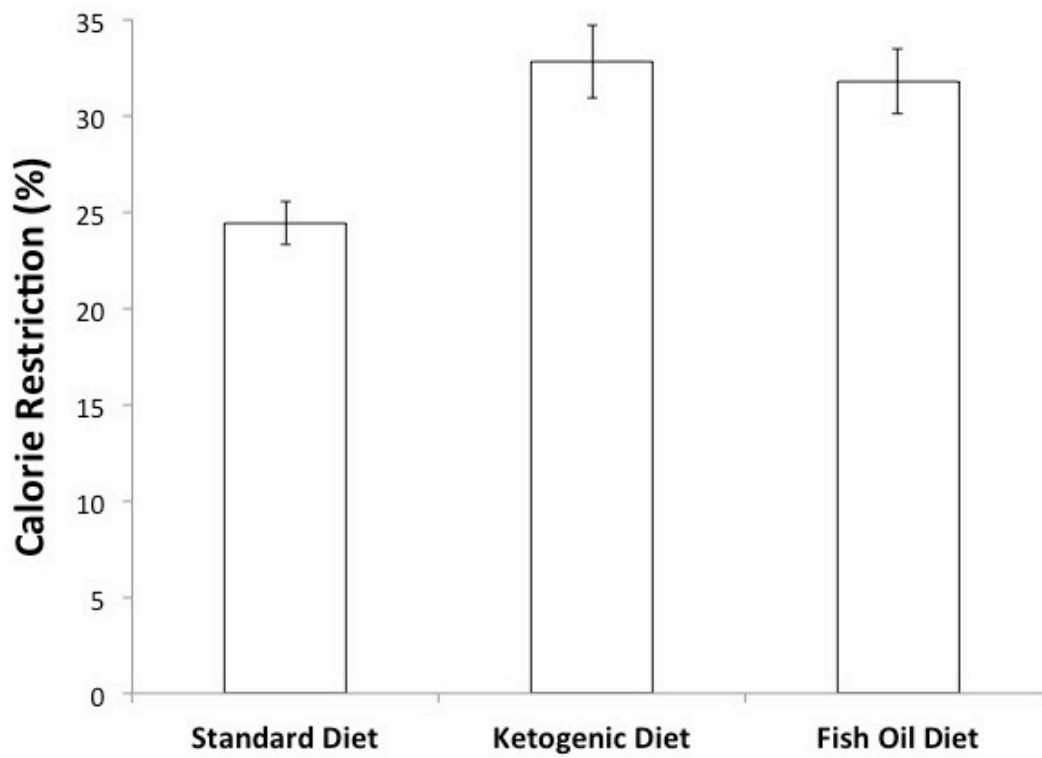
# Weight Gain in Juvenile EL mice



**Figure 5.** Effect of calorie restriction on mouse body weights in standard chow diet, ketogenic diet, and a fish oil-supplemented chow diet. Three dietary groups were calorie restricted to lose 20% of their original body weight, which corresponds to a final body weight of  $23.3 \pm 0.2$  g. To reach a 20% body weight reduction, the Ketogenic Diet and Fish Oil Diet groups require a greater amount of calorie restriction ( $\sim 32.5\%$ ) compared to the Standard Diet (24%).

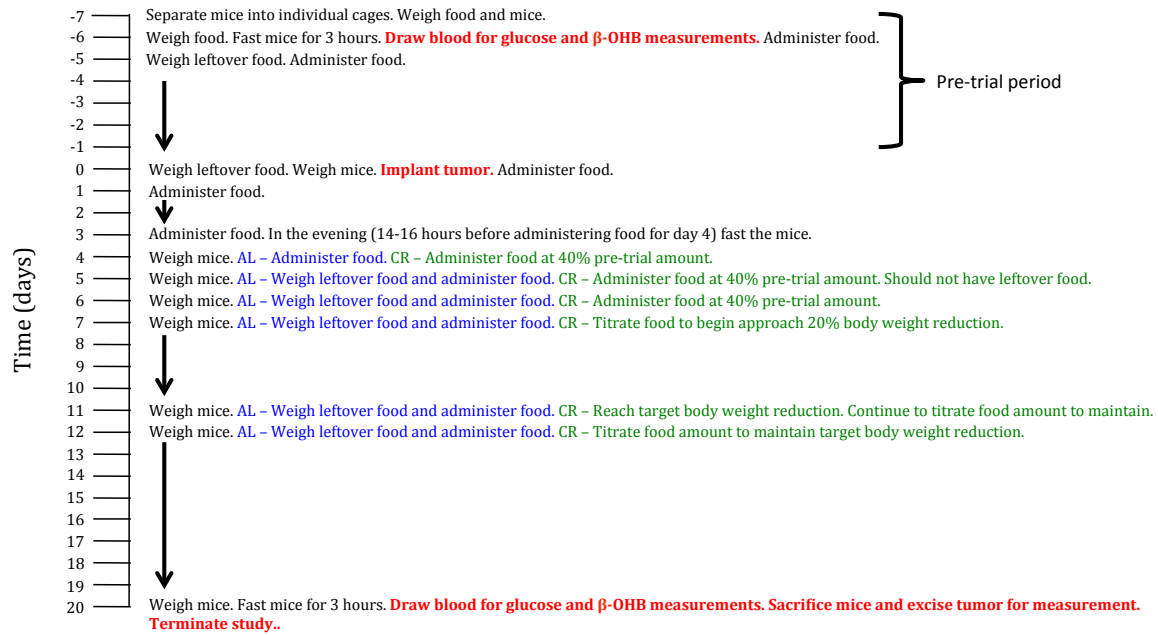


### Degree of Calorie Restriction Necessary to Lose 20% Bodyweight for Standard, Ketogenic, and Fish Oil Diets in Mice



**Figure 6.** Sample dietary plan to study the effect of calorie restriction on tumor growth. In this sample plan, the effect of a 20% body weight reduction on the growth of a tumor is compared to an *ad libitum* (AL) fed group. The pretrial period lasts for 7 days. During the pretrial period, blood is drawn to establish a metabolic baseline and food intake is tracked. The study period commences on day 0. Diets are initiated on day 4 to ensure the tumor is growing. The CR group will have their calorie intake titrated to reach a body weight reduction of 20% after one week. The body weight reduction is maintained until the study is terminated on day 20. Blood is drawn to evaluate the effects that the diets have on blood glucose and  $\beta$ -hydroxybutyrate levels.

## Sample Dietary Plan to Study the Effects of Calorie Restriction on Tumor Growth



**Figure 7.** Sample dietary plan to study the effect of the ketogenic diet on autistic behavior. In this sample plan, the effect of a ketogenic diet on autistic behavior is compared to a standard mouse chow diet. The pretrial period lasts for 7 days. During the pretrial, behavioral testing occurs on days -3, -2, and -1 to establish a behavioral profile baseline. Blood is drawn to establish a metabolic baseline and food intake is tracked. The study period commences on day 0, with a 16-hour overnight fast. The feeding of the ketogenic diet and the study lasts for 4 weeks. On the last three days of the study, behavior is tested to assess the effects of the ketogenic diet on autistic behavior. Blood is drawn to evaluate the effects that the diets have on blood glucose and  $\beta$ -hydroxybutyrate levels.

## Sample Dietary Plan to Study the Effects of the Ketogenic Diet on Autistic-Like Behavior



## CHAPTER THREE

### *Influence of a ketogenic diet, fish-oil, and calorie restriction on plasma metabolites and lipids in C57BL/6J mice*

#### INTRODUCTION

Mounting evidence suggests that dietary intake can influence the prognosis of a broad range of diseases, including epilepsy, autism, cancer, Alzheimer's disease, and cardiovascular disease (Baranano & Hartman, 2008; Kashiwaya et al., 2013; Schroeder et al., 2010; Stafstrom & Rho, 2012; World Health Organization. & Food and Agriculture Organization of the United Nations., 2003). Popular diets to treat these conditions include the ketogenic diet, low-glycemic index treatment diet, fish-oil supplemented diets, and calorie restricted diets. There is continued debate and interest on how dietary composition and quantity affects body weight, blood lipid profile, and glycemic control (Hooper et al., 2012a; Hooper et al., 2012b; Schwarzfuchs et al., 2012; Shai et al., 2008; Volek et al., 2009). In order to effectively utilize dietary therapy to treat disease, it is important to understand how different treatment modalities could affect plasma metabolites, such as glucose and ketone bodies, and the overall health and vitality of the subjects.

Plasma glucose and ketone levels are important prognosticators of dietary efficacy for a variety of neurological and non-neurological disorders (Greene et al., 2003; Kashiwaya et al., 2013; Mantis et al., 2009; Maurer et al., 2011; Seyfried et al.,

2003; Seyfried et al., 2011; Veech, 2004). In epilepsy, studies revealed that the efficacy of the restricted ketogenic diet relies on how well the diet lowers blood glucose and elevates blood ketone levels (Bough et al., 1999; Eagles et al., 2003; Mantis et al., 2004). Similarly, preclinical and clinical studies have shown a positive effect of increased circulating ketone levels and reduced glucose levels in disease outcome in cancer (Fine et al., 2012; Seyfried et al., 2003; Zhou et al., 2007; Zuccoli et al., 2010). In healthy individuals, however, it is unclear whether reduced glucose levels and increased ketone levels improves health, though it may be associated with metabolic disease resistance (Joo et al., 2010).

Conflicting results on blood lipid and glucose levels were reported in humans and mice on the ketogenic diet. In adults on the ketogenic diet, triglyceride levels remain unchanged or are lowered, whereas cholesterol levels remain unchanged, are lowered, or are increased (Dashti et al., 2006; Klement et al., 2013; Westman et al., 2002; Yancy et al., 2004). The ketogenic diet increases plasma triglyceride and cholesterol levels in children (Kwiterovich et al., 2003). Fasting glucose in both adults and children remains unchanged or is lowered on the ketogenic diet (Brehm et al., 2003; De Vivo, 1983; Klement et al., 2013; Meckling et al., 2004; Tandler et al., 2007; Volek et al., 2003), whereas the blood ketones are consistently increased (Gilbert et al., 2000). In mice, the ketogenic diet decreases plasma triglyceride levels while increasing cholesterol levels (Badman et al., 2009; Kennedy et al., 2007). Increased, decreased, or no change in plasma glucose levels were reported in mice on the ketogenic diet (Kennedy et al., 2007; Mantis et al., 2004; Seyfried et al., 2003). As in children, plasma ketone levels are

consistently elevated in mice on ketogenic diets (Kennedy et al., 2007; Mantis et al., 2004).

Supplementation with fish-oil leads to more consistent effects on blood lipid and glucose levels, compared to the ketogenic diet. Blood triglyceride levels decrease, whereas total cholesterol levels remain unchanged in humans (Kaul et al., 2008; Mori et al., 1999; Mori et al., 2000; Woodman et al., 2002; Zulyniak et al., 2013). Fasting glucose levels are unchanged in non-diabetic individuals, but are increased in type-2 diabetic individuals that supplement with fish-oil (Mori et al., 1999; Mori et al., 2000; Woodman et al., 2002). Blood ketone levels remain unchanged after supplementation with fish-oil (Dagnelie et al., 1994). Fish-oil supplemented and derived diets in mice reduce total plasma cholesterol and either decrease or have no effect on plasma triglyceride levels (Degirolamo et al., 2010; Murali et al., 2012; Yang et al., 2011). Fasting glucose in mice remains unchanged or is decreased in mice given fish-oil supplemented or derived diets (Mori et al., 2007; Murali et al., 2012; Yang et al., 2011). Hepatic ketogenesis increased in rats fed a fish-oil-supplemented diet, due to decreased lipogenesis and increase in fatty acid oxidation (Wong et al., 1984).

Calorie restriction is a long-studied dietary modality that is often used in conjunction with the ketogenic diet to treat a variety of neurological disorders (Maalouf et al., 2009), and is used as a prophylactic for cardiovascular disorders and other pathologies (Hursting et al., 2010; Weiss & Fontana, 2011; Wu et al., 2008). Calorie restriction in humans leads to consistent reductions in total cholesterol and



triglyceride levels (Fontana et al., 2004; Fontana et al., 2007; Holloszy & Fontana, 2007), while blood glucose levels are consistently lowered (Fontana et al., 2004; Holloszy & Fontana, 2007) and plasma ketone body levels are increased (Johnson et al., 2007; Knopp et al., 1991). In mice, calorie restriction leads to either a decrease or no change in plasma total cholesterol and triglyceride levels (Kennedy et al., 2007; Mahoney et al., 2006). Calorie restriction in mice generally leads to a decrease in plasma glucose levels and increase in plasma ketone body levels (Greene et al., 2001; Mahoney et al., 2006; Mantis et al., 2004; Seyfried et al., 2003), although there are reports showing glucose levels can remain unchanged with calorie restriction (Kennedy et al., 2007), and ketone body levels remaining unchanged or even decreasing with calorie restriction (Anson et al., 2003; Kennedy et al., 2007).

To assess the effects of diet on plasma glucose and ketone levels, along with their effect on hormones and lipids, I evaluated the influence of a standard mouse chow diet, a ketogenic diet, and a fish-oil supplemented diet under *ad libitum* feeding conditions in the inbred C57BL/6J (B6) mouse strain. These diets can be used to treat diseases and disorders, and it remains unclear if the therapeutic efficacy of the diets is related to their composition or to a modest calorie reduction (Bough & Rho, 2007; Cullingford, 2004). The three diets were also evaluated under calorie-restricted conditions. These diet types have been used extensively in the literature, with the ketogenic diet being used to treat epilepsy and other neurological disorders and the fish-oil supplemented diet being used to treat

cardiovascular dysfunction and inflammatory disease (Calder, 2006; McEvoy et al., 2012; Simopoulos, 2002a; Simopoulos, 2002b; Stafstrom & Rho, 2012; Vijay-Kumar et al., 2011). I found differences in plasma metabolite profiles between the standard diet, ketogenic diet, and fish-oil supplemented diet, when fed *ad libitum*. However, these differences were mostly minimized when administered under calorie restriction, which suggests that biomarkers of health are improved in calorie-restricted diets regardless of macronutrient composition.

## MATERIALS AND METHODS

### Mice

The C57BL/6J (B6) mice were obtained originally from Jackson Laboratory (Bar Harbor, ME). The mice were maintained in the Boston College Animal Care Facility. Adult male mice (120 days of age) were used and housed individually in a temperature-regulated room at 22°C and kept on a 12-hour light-dark cycle. Food was provided either *ad libitum* (AL) or restricted to reduce body weight by 20% (CR). Water was provided *ad libitum* to all mice. All animal procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Boston College Institutional Animal Care and Use Committee (IACUC).

## Diets

All mice were fed *ad libitum* with standard rodent chow (Prolab RMH 3000; PMI LabDiet, Richmond, IN, USA) during the first seven days of the study (pre-trial period). This is the standard mouse pellet diet, which contains a balance of vitamins and minerals. The standard diet (SD) was prepared by mixing 1 g of powdered standard mouse diet with 1 mL of water to form a paste. The lard-based ketogenic diet (KD) was prepared by the manufacturer (Zeigler Bros. Inc., Gardners, PA, USA), and has a full complement of vitamins and minerals prepared specifically for rodents. The fish-oil supplemented diet (FO) was prepared by mixing 3g of powdered standard mouse diet with 1g of CVS Fish Oil to form a crude paste. The composition of the diets is given in **Table 3**. All diets were placed into 25 mL beakers, which were previously filled halfway with solid baseplate wax, to allow mice free access to the food.

## Dietary Treatment

After the seven-day pretrial period, mice were assigned to one of six groups (n = 4 mice/group): 1) standard mouse diet fed AL (SD-UR), 2) standard mouse chow diet fed CR to achieve a 20% body weight reduction (SD-R), 3) the lard-based ketogenic diet fed AL (KD-UR), 4) the lard-based ketogenic diet fed CR to achieve a 20% body weight reduction (KD-R), 5) the fish-oil supplemented diet fed AL (FO-UR), and 6) the fish-oil supplemented diet fed CR to achieve a 20% body weight reduction (FO-R).

Body weights and food intake measurements were taken daily three hours after lights-on. Based on food intake and body weight during the pretrial period, food in the CR groups was restricted to achieve a 20% reduction in body weight. I used body weight as the endpoint for CR, as the Seyfried laboratory has previously demonstrated that body weights are a more stable and consistent variable than food intake, which changes significantly on a daily basis in *ad libitum* fed mice (Greene et al., 2001; Mahoney et al., 2006; Mantis et al., 2004).

The study period lasted a total of 32 days. After the 7-day pretrial period, mice were fasted for 16 hours on day 0, before initiating their respective diets. By day 7 of the dietary treatment, body weight and food intake stabilized. Therefore, all calculations involving body weight and food intake were taken from days 7-32 of the study. Metabolizable energy intake was calculated according to the manufacturers' measurements of metabolizable energy content (**Table 3**).

#### Collection of Plasma

After a 3-hour fast on day 32 (during the light cycle), mice were anesthetized with isoflurane, and plasma was obtained by collecting blood into heparinized tubes through the retro-orbital sinus. Plasma was collected by centrifuging blood at 6,000 x g for 10 minutes at 4°C and was stored at -80°C until analysis.

### Measurement of Glucose, $\beta$ -hydroxybutyrate, and Hormones

Glucose was measured spectrophotometrically using the Trinder Assay (Sigma-Aldrich, St. Louis, MO, USA).  $\beta$ -hydroxybutyrate was measured enzymatically using a modification of the Williamson *et al.* procedure (Williamson *et al.*, 1962). Plasma insulin was measured by rat/mouse insulin ELISA (Millipore, Billerica, MA, USA), and plasma adiponectin was measured by mouse adiponectin ELISA (Millipore).

### Measurement of Lipids

The plasma lipid content of the triglycerides, cholesterol, free fatty acids, cholesteryl ester, phosphatidylcholine, lyso-phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin were analyzed, as previously described (Baek *et al.*, 2009). Briefly, lipids were extracted by adding chloroform (C) and methanol (M) to plasma in a ratio of 30:60:8 (C:M:plasma by volume). The lipid extract was added to DEAE-Sephadex (A-25, GE Healthcare, Piscataway, NJ) to separate the neutral and acidic lipid fractions, as previously described (Macala *et al.*, 1983). Neutral lipids were eluted from the column with C:M:dH<sub>2</sub>O at 30:60:8 by volume, and dried by rotary evaporation. The acidic lipids, which contain a portion of the free fatty acids, were eluted with C:M:0.8M sodium acetate at 30:60:8 by volume, and dried by rotary evaporation.

To quantify the lipids, an equivalent of 3  $\mu$ L of plasma was spotted per lane on 10 x 20 cm Silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) using a

Camag Linomat II auto-TLC spotter (Camag Scientific Inc., Wilmington, NC, USA).

The plates were developed in a solvent system that contained C:M:acetic acid:formic acid:dH<sub>2</sub>O (70:30:12:4:2 by volume) up to 4.5 cm, and then run in a second solvent system containing hexane:isopropyl ether:acetic acid (65:35:2 by volume) up to 10 cm, as previously described (Macala et al., 1983; Mahoney et al., 2006). Lipid bands were visualized by charring with 3% cupric acetate in 8% phosphoric acid solution and were scanned using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA, USA). The concentration of each individual lipid was calculated from a standard curve. To measure free fatty acids, which separate into both neutral and acidic lipid fractions, an equivalent of 3  $\mu$ L of plasma from the neutral and acidic lipid portion was combined and spotted, as described above.

### Statistical Analyses

Group size was determined through power analysis using G\*Power 3 statistical software (Faul et al., 2007).  $\alpha$  error probability and  $\beta$  error probability were set to a maximum of 0.01, and effect size was conservatively estimated from previous data (Kennedy et al., 2007; Mahoney et al., 2006; Mantis et al., 2004). This yielded an *n* of 4 mice per group for six groups, with an actual power of 0.9927 and a critical F value of 4.248. All other statistical analyses were performed using SPSS Software (IBM SPSS Statistics, Version 20). All values are presented as mean  $\pm$  standard error of the mean (SEM). One-way ANOVA was used to evaluate differences between dietary groups. Bivariate linear regression analysis with

ANOVA was used to independently assess the predictive strength of select independent variables on final body weight, plasma glucose,  $\beta$ -hydroxybutyrate, and triglycerides, regardless of dietary treatment. Pearson bivariate correlation analysis was used to determine the relationship between body weight, food intake, plasma glucose,  $\beta$ -hydroxybutyrate, insulin, adiponectin, and lipid levels.

## RESULTS

Dietary modification as a treatment modality is subject to variability, as investigators often use different approaches to implement the diets, which hinders study reproducibility. To improve reproducibility and evaluate the merits of each diet, all mice were fasted for 16 hrs prior to initiating the diets. This allows for a proper evaluation of the diets, as the mice will have the same metabolic set point. Also, the influence of the previously administered standard rodent chow on the animal's metabolism will be minimized (Reed et al., 1991). I have also observed that fasting mice before initiating diets also serves to limit the amount of self-restriction that mice impose when switched to an unfamiliar diet (personal observations).

### Body Weight and Metabolizable Energy Intake

All CR mice lost approximately 20% of their initial body weight (**Figure 8, Table 4**). All mice tolerated the diets well and appeared healthy and vigorous at

the end of the study. Activity levels were comparable in all groups, and lethargy was not present in any mouse.

Body weights stabilized after approximately 7 days following diet implementation (**Figure 8**), whereas calorie intake stabilized after 8-10 days (data not shown). While the SD-UR group maintained body weight throughout the study, the KD-UR group gained approximately 11% body weight during the 32-day period, despite consuming approximately 10% less in calculated daily calories compared to SD-UR (**Table 4**). The FO-UR group lost approximately 11% body weight during the 32-day period, and this occurred with a 12% reduction in calorie intake compared to SD-UR. While body weight was reduced by 20% in each CR group relative to the *ad libitum* control group, the amount of CR necessary to achieve this weight loss depended on the macronutrient composition of the diet. Mice fed the higher-fat diets (KD-R and FO-R) required a calorie reduction of approximately 33%, whereas mice fed the normal chow diet (SD-R) required a 24% calorie reduction to achieve the 20% body weight loss. The FO-UR group consumed 700 mg of fish oil per day, whereas the FO-R group consumed 550 mg per day.

#### Influence of Diet on Plasma Metabolites and Hormones

Glucose levels were reduced in all three CR groups compared to the levels in the SD-UR group and in their respective unrestricted diets (**Table 5**). Glucose levels were also lower in the FO-UR group than in the SD-UR group, though this might reflect the differences in body weights. The blood glucose levels in the FO-UR group



were similar to those in the KD-R group. Blood glucose levels were significantly higher in the KD-UR group than in the SD-UR group.

$\beta$ -hydroxybutyrate (major circulating ketone) levels were increased in all the CR groups compared to their respective *ad libitum* diet groups, with the KD-R group exhibiting the highest levels of ketones (**Table 5**). KD-UR and FO-UR groups had significantly elevated ketone levels compared to the SD-UR group.

Plasma insulin levels were assessed from the mice after a 3-hour fast, which along with fasting blood glucose levels comprise a homeostatic model assessment of insulin resistance (HOMA-IR) value and is indicative of insulin sensitivity (Lansang et al., 2001; Maeda et al., 2002; Trout et al., 2007). Low HOMA-IR values indicate increased insulin sensitivity. Insulin levels were lower in all CR groups than in their comparable *ad libitum* groups, which indicates high insulin sensitivity (**Table 5**). While insulin levels were similar in the SD-UR and KD-UR groups, insulin levels were lower in FO-UR group and comparable to those in the CR groups. The high insulin sensitivity mirrored the lower plasma glucose levels in FO-UR mice. We found that plasma glucose and insulin levels were highly correlated across all groups (**Table 6**). Adiponectin levels were highest in the CR groups, with SD-R and KD-R having significantly elevated levels, and FO-R trending toward increased levels (**Table 5**). The levels of adiponectin were similar across all unrestricted diets.

### Influence of Diet on Plasma Lipids

The plasma lipid distribution was examined using HPTLC (**Figure 9**). The changes in plasma triglyceride levels were generally correlated with the changes as plasma glucose levels, with the CR groups exhibiting the lowest levels of circulating triglycerides and glucose (**Table 5**). The KD-UR group had the highest levels of triglycerides, whereas the FO-UR group exhibited triglyceride levels that were similar to the levels measured in the CR groups. Total free cholesterol levels were reduced in SD-R and KD-R groups compared to their unrestricted controls, although cholesterol levels in KD-R group were similar to those in the SD-UR group. The cholesterol levels in both fish-oil groups were similar to those seen in to SD-UR. Cholesteryl ester levels follow a similar pattern to free cholesterol. While cholesteryl ester levels were not significantly decreased in SD-R compared to SD-UR, the KD groups had the highest levels of cholesteryl esters, with KD-UR having significantly higher levels compared to KD-R. Cholesteryl ester levels were unchanged in the fish-oil groups, and were similar to those in the SD-UR group. Free fatty acid levels (FFA) were higher in SD-R and KD-R groups than in their respective UR groups.

Phosphatidylcholine (PC) levels were reduced in the SD-R and KD-R groups compared to their respective unrestricted diets, but were higher in the KD group. The fish-oil groups had similar PC levels. Lyso-phosphatidylcholine (LPC) levels were reduced slightly in the SD-R group, and were similar across the rest of the groups (**Table 5**). Plasma phosphatidylethanolamine (PE) levels were lowest in the

fish-oil groups, and were significantly reduced in the SD-R and KD-R groups compared to the unrestricted diets, although the KD groups had the highest overall levels of PE. Sphingomyelin (SM) levels were highest in the KD groups, and were significantly reduced in SD-R compared to SD-UR. There was no difference between the fish-oil groups, and they were comparable to the levels seen in SD-UR. Plasma ceramide and cerebroside levels were also evaluated, however they were present only in trace amounts and were not quantified (**Figure 9**).

#### Major Predictors of Body Weight, Glucose, Ketone, and Triglyceride Levels

I pooled the dietary groups to independently assess the predictive strength of independent variables (**Figure 10**). The assumptions of bivariate linear regression with ANOVA were met, according to established criteria (Lang & Secic, 1997). The strongest independent predictor (highest coefficient of determination) for final body weight, plasma glucose, ketone, and triglyceride levels is displayed in **Figure 10** for each of the variables assessed. I found that while calories and ketones moderately predict body weight, blood glucose level ( $R^2 = 0.850$ ; 95% CI =  $1.000 \pm 0.186$ ;  $t_{22} = 11.157$ ;  $y = 16.798 + 1.000x$ ) was the strongest predictor of final body weight during the study. The amount of dietary fat and dietary carbohydrates consumed were not significant indicators of final body weight.

While I found that circulating glucose levels was the strongest predictor of final body weight, calorie intake was the most significant predictor of blood glucose level ( $R^2 = 0.500$ ; 95% CI =  $1.304 \pm 0.5777$ ;  $t_{22} = 4.687$ ;  $y = -6.390 + 1.304x$ ). Dietary

fat intake also moderately predicted plasma glucose levels, whereas dietary carbohydrate intake was not a significant predictor of glucose levels.

The most important predictor of circulating ketones was reduced calorie intake ( $R^2 = -0.740$ ; 95% CI =  $-0.399 \pm 0.105$ ;  $t_{22} = -7.904$ ;  $y = 6.301 - 0.399x$ ). While lowering calories had the greatest effect on increasing ketone levels, lowering dietary carbohydrate intake also had a significant, but moderate influence ( $R^2 = 0.384$ ; 95% CI =  $-0.591 \pm 0.332$ ;  $t_{22} = -3.700$ ;  $y = 2.018 - 0.591x$ ) on ketone levels. Glucose level was a weak predictor of ketone levels, and surprisingly, dietary fat intake was not a significant predictor of circulating ketone levels.

Circulating glucose levels strongly predicted circulating triglyceride levels ( $R^2 = 0.883$ ; 95% CI =  $4.223 \pm 0.672$ ;  $t_{22} = 12.870$ ;  $y = -22.631 + 4.233x$ ). Calorie and dietary fat intake moderately predicted triglyceride levels, whereas ketone levels were a weak predictor of triglyceride levels. Dietary carbohydrate intake was not a predictor of circulating triglyceride levels.

## DISCUSSION

I found that the macronutrient composition of a diet greatly affected the plasma metabolite profile of C57BL/6J mice when administered *ad libitum*. A ketogenic diet administered *ad libitum* promoted high glucose levels, weight gain, and a hyperlipidemic profile in mice, whereas fish-oil supplementation decreased bodyweight, glucose levels, and yielded a normolipidemic profile. Administration of

these diets under CR led to weight loss, increased insulin sensitivity, decreased glucose levels, increased ketone levels, and promoted a normolipidemic lipid profile. When administered under CR, the influence of the composition of the diet on the metabolic profiles of the mice was minimized, yielding similar metabolic profiles across all groups. Thus, a restriction of total energy intake can correct weight gain or a hyperlipidemic profile from these dietary therapies in B6 mice.

I provided additional evidence that the KD-UR does not lead to long-term weight loss in mice when they are fasted prior to diet initiation (Borghjii & Feinman, 2012; Mantis et al., 2004; Seyfried et al., 2003). This work supports the notion that “a calorie is not a calorie”, as the KD-UR group gained approximately 10% body weight despite consuming 10% less in metabolizable calorie intake, compared to SD-UR. However, these results challenge the metabolic advantage theory of the KD-UR, which states that isocaloric diets low in carbohydrates lead to greater weight loss compared to isocaloric diets of different composition (Feinman & Fine, 2004). This was unsurprising, given that dietary fat is metabolized more efficiently than are dietary carbohydrates and proteins (Jequier, 2002). The seeming discrepancy between metabolizable calorie intake and body weight is most likely due to differences in energy utilization between the different dietary groups, including factors such as obligatory thermogenesis, microbial fermentation, diet induced thermogenesis, and basal metabolism (including energy expenditure) (MacLean et al., 2003).

In addition to the increased body weight under KD-UR, the plasma profile of the KD-UR group suggests that the diet is unhealthy (Ansar et al., 2011; Carlson & Bottiger, 1972; Hokanson & Austin, 1996; Kannel et al., 1979). The plasma profile of the KD-UR mice is similar to a previously published report utilizing a high-fat, high-sucrose diet in the same strain of mice (Kennedy et al., 2007). The atherogenic lipids triglycerides, cholesterol, and cholesteryl esters were highest in KD-UR group. A KD-UR diet also elevated these lipids in children with epilepsy (Kwiterovich et al., 2003). The presence of high circulating glucose levels in the KD-UR group, which has also been reported in other studies of rodents on high-fat, low-carbohydrate diets (Bielohuby et al., 2013; Ellenbroek et al., 2014), could further increase the risk of atherogenesis, as glucose can contribute to endothelial dysfunction (Beckman et al., 2002; Kanter et al., 2007). Glucose can be raised in high-fat diets devoid of carbohydrates with excess calorie intake through gluconeogenesis, which occurs indirectly through oxidation of fatty acids to acetyl-CoA (Weinman et al., 1957). The KD-UR group also had the highest levels of PC, LPC, PE, and SM. It has been suggested that a PC to free cholesterol ratio (mol/mol) of less than 1 is a risk factor for atherosclerosis (Kuksis et al., 1983). The calculated PC/cholesterol ratios in all of the groups (both restricted and unrestricted) were similar, and ranged from 1.5-1.75 (data not shown). LPC levels have been shown to be positively associated with obesity (Barber et al., 2012). PE levels are suggested to be positively associated with atherosclerotic complications (Kunz & Stummvoll, 1971). SM in plasma is associated with an increased risk of coronary artery disease (Jiang et al., 2000;

Schlitt et al., 2006). Our data are consistent with the observations for LPC, PE, and SM. These findings, together with our observations in B6 mice, indicate that consumption of the KD in unrestricted amounts can have adverse effects on health-related biomarkers.

Administration of the KD in restricted amounts mitigated the hyperlipidemic plasma profile of the B6 mice. In addition to lowering triglycerides, cholesterol, phospholipids (PC, LPC, and PE), and glucose, the KD-R also lowered fasting insulin levels. Reduced levels of circulating insulin is antiatherogenic (Salt, 2013). Plasma ketone levels were also highest in the KD-R group. Ketones are therapeutic against a variety of neurological diseases and cancer (Gasior et al., 2006b; Maalouf et al., 2009; Rho & Sankar, 2008; Shimazu et al., 2013; Stafstrom & Rho, 2012). It is well known that ketones can replace glucose as an energy metabolite and can protect the brain from hypoglycemia (Drenick et al., 1972; VanItallie & Nufert, 2003; Veech, 2004). Our previous findings, in conjunction with the results in the present study, suggest that the therapeutic efficacy of the KD for epilepsy and brain cancer will likely be best when the diet is administered in restricted than unrestricted amounts (Mantis et al., 2004; Seyfried et al., 2003; Zhou et al., 2007).

While the KD-UR increased body weight and was associated with a hyperlipidemic plasma profile, it was interesting to find that the FO-UR diet caused changes in body weight and plasma metabolites that were similar to those seen in the CR groups. I cannot completely rule out that these results are a consequence of decreased food palatability in the FO-UR group, as the FO-UR group had reduced

calorie intake compared to SD-UR. The FO-UR group, however, consumed a similar number of calories as the KD-UR group after the fast ( $16.9 \pm 2.3$  (FO-UR) kilocalories versus  $17.0 \pm 0.3$  (KD-UR) kilocalories) and throughout the study, and did not have observable aversion to the food. I hypothesized that increased levels of the adipokine adiponectin might be responsible for the increased insulin sensitivity and decreased body weight in the FO-UR group despite feeding *ad libitum*, since supplementation of fish-oil is associated with increased levels of adiponectin (Flachs et al., 2006; Neschen et al., 2006). Interestingly, I found that adiponectin was not elevated in the FO-UR group, suggesting that adiponectin by itself was not responsible for the increase in insulin sensitivity or the CR-like profile of the FO-UR mice. Omega-3 fatty acids and their metabolites can produce changes in the activity of peroxisome proliferator-activated receptor (PPAR)  $\alpha$  and  $\gamma$ , along with directly binding to and modulating the activity of sterol receptor element binding proteins (SREBP), which contribute to modulating lipid and glucose metabolism (Davidson, 2006; Deckelbaum et al., 2006). Omega-3 fatty acids and their metabolites (epoxide derivatives, docosanoids, resolvins, and neuroprotectins) reduce triglyceride levels, adiposity, and inflammation while improving insulin sensitivity in rodents and humans (Lombardo & Chicco, 2006; Oh et al., 2010). Thus, the observed plasma profile of the FO-UR mice that mimicked the CR groups were likely due to a multitude of mechanisms regulated by omega-3 fatty acids that are still not fully elucidated.



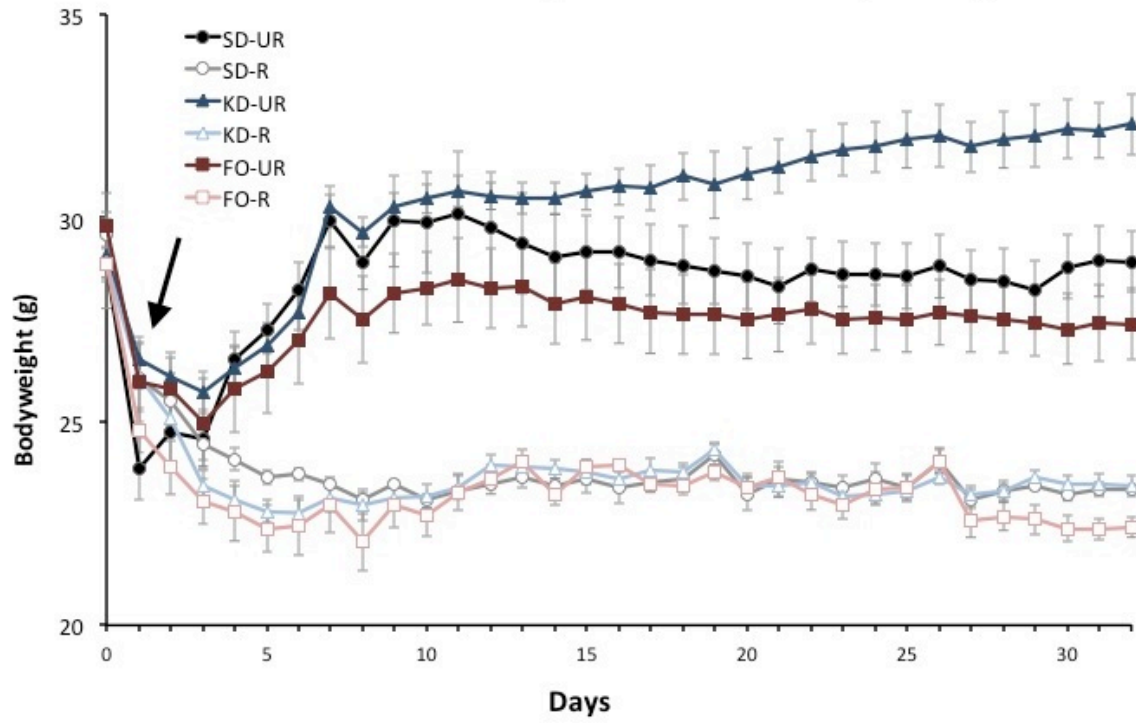
All of the dietary groups were combined in a post-hoc analysis to examine the relationship among body weight, dietary intake, and plasma profile. My findings indicated that blood glucose was the strongest predictor of body weight in mice and was highly correlated with body weight. This observation is consistent with previous studies in humans, though the human studies did not review dietary intake (Folsom et al., 1996; Qian et al., 2010). I found that intake of specific macronutrients was not predictive of final bodyweight. This observation questions the utility of advocating for diets devoid in specific macronutrients to control body weight (Shai et al., 2008; Shai & Stampfer, 2008). In terms of predicting glucose levels, total calorie intake was only moderately predictive suggesting that multiple factors modulate blood glucose levels in mice. These findings suggest that restriction of total calorie intake will be more effective for reducing glucose levels and body weight in mice than will be the restriction of any particular macronutrient under *ad libitum* feeding. The reduction of total calories was strongly predictive of elevated ketone levels. Reduced carbohydrate intake contributed moderately to increased ketone levels. From a physiological perspective, given that the human body has a large reservoir of lipid stores and a small reservoir of glycogen stores (~150 g), it should not be surprising that restriction of energy intake and carbohydrates will quickly diminish glycogen stores, leading to beta-oxidation of lipids for energy (Flatt, 1987). Dietary strategies that promote beta-oxidation of lipids, through either restriction of overall energy intake or severe carbohydrate restriction, would promote ketosis. Since circulating glucose strongly predicted plasma triglyceride

levels, dietary strategies that lower blood glucose levels should have significant effects on promoting cardiovascular and overall health (Ford, 2004; O'Keefe & Bell, 2007).

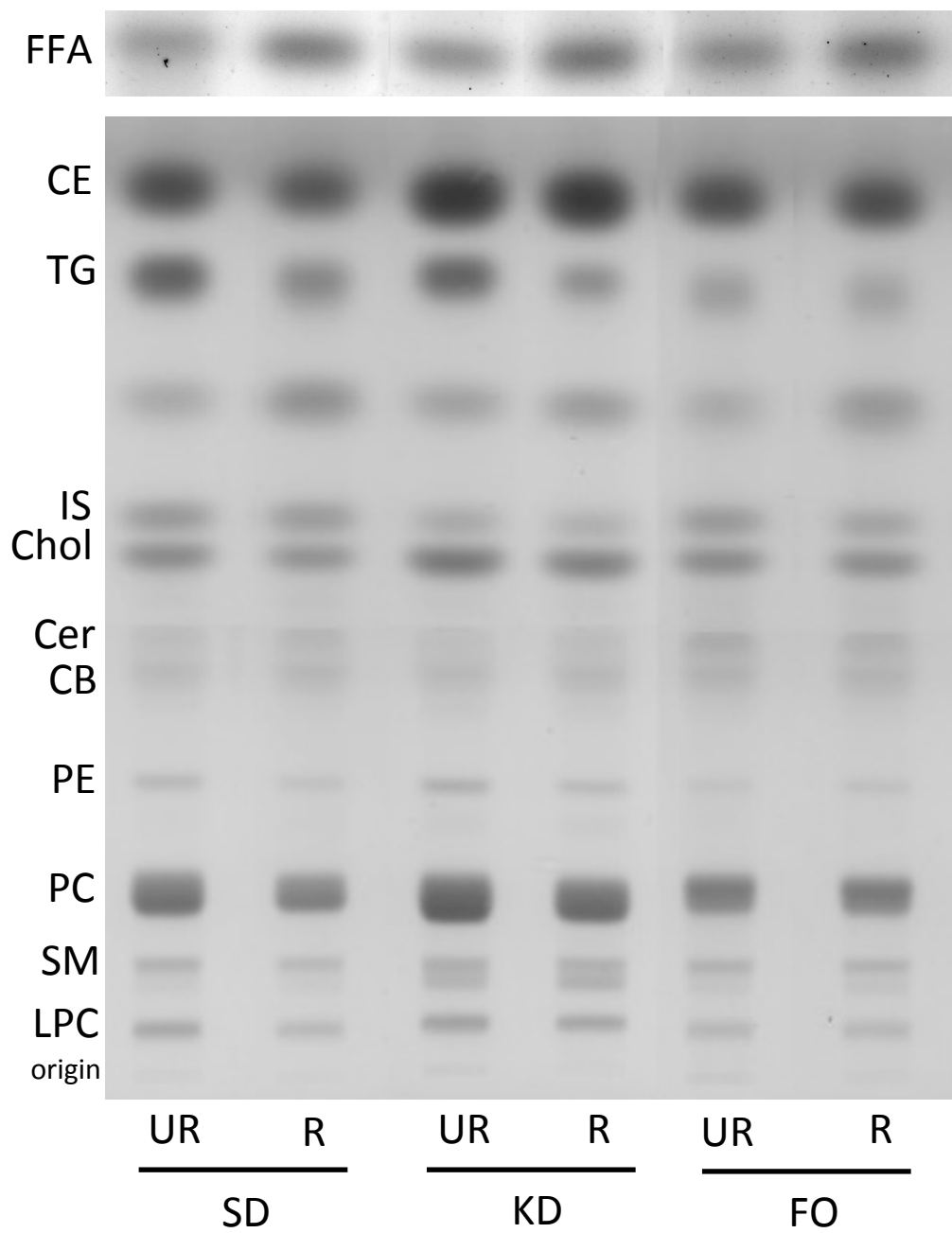
The macronutrient composition of various diets plays an important role when the diet is fed *ad libitum*, as our data showed for body weight, hormones, and plasma metabolites. CR, however, has a dominant and independent effect on body weight, hormones, and plasma metabolites, compared to macronutrient composition. These data suggest that CR may be more appropriate for improving health outcomes than shifting macronutrient ratios, especially when using dietary therapy to treat metabolic diseases (Mantis et al., 2004; Seyfried et al., 2003).

**Figure 8.** Influence of dietary regimen on body weight. Weights are expressed as means  $\pm$  SEM. Arrow represents initiation of respective diets after a 16 hour fast. *SD-UR*, standard diet unrestricted; *SD-R*, standard diet restricted; *KD-UR*, ketogenic diet unrestricted; *KD-R*, ketogenic diet restricted; *FO-UR*, fish-oil supplemented diet unrestricted; *FO-R*, fish-oil supplemented diet restricted.

## Influence of Dietary Intake on Body Weight

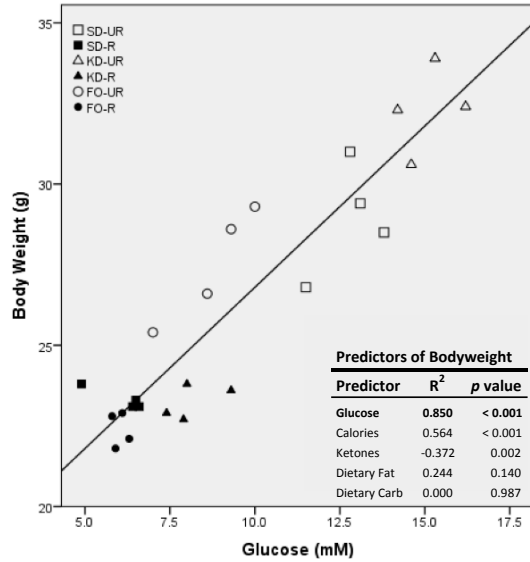


**Figure 9.** HPTLC plate of plasma lipids in mice under Standard Diet (SD), Ketogenic Diet (KD), and Fish-Oil Diet (FO) under both unrestricted (UR) and restricted (R) feeding conditions. *FFA*, free fatty acids; *CE*, cholesteryl ester; *TG*, triglycerides; *IS*, internal standard (oleyl alcohol); *Chol*, cholesterol; *Cer*, ceramide; *CB*, cerebroside; *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *SM*, sphingomyelin; *LPC*, lyso-phosphatidylcholine. All lipid values are quantified in Table 3, except for ceramides and cerebroside, since there are only trace amounts present.

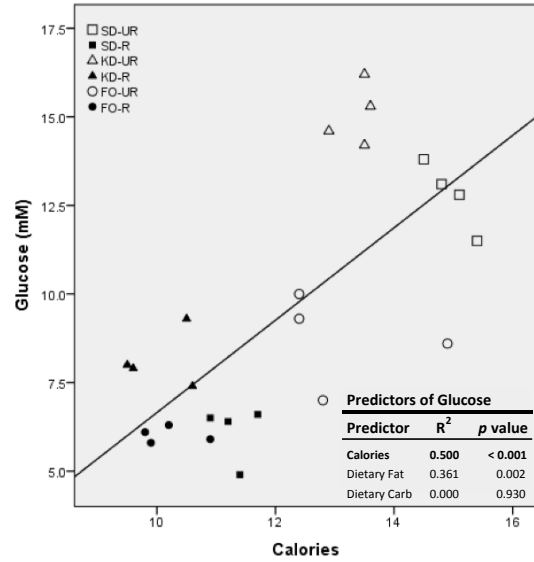


**Figure 10.** Linear regression analyses of body weight, glucose,  $\beta$ -hydroxybutyrate, and triglyceride levels in mice. The strongest predictor (highest coefficient of determination) for each dependent variable is plotted. Irrespective of diet, glucose levels most strongly predict body weight and triglyceride levels, calories most strongly predict glucose and ketone levels, and dietary fat intake most strongly predicts cholesterol levels. *SD-UR*, standard diet unrestricted; *SD-R*, standard diet restricted; *KD-UR*, ketogenic diet unrestricted; *KD-R*, ketogenic diet restricted; *FO-UR*, fish-oil supplemented diet unrestricted; *FO-R*, fish-oil supplemented diet restricted.

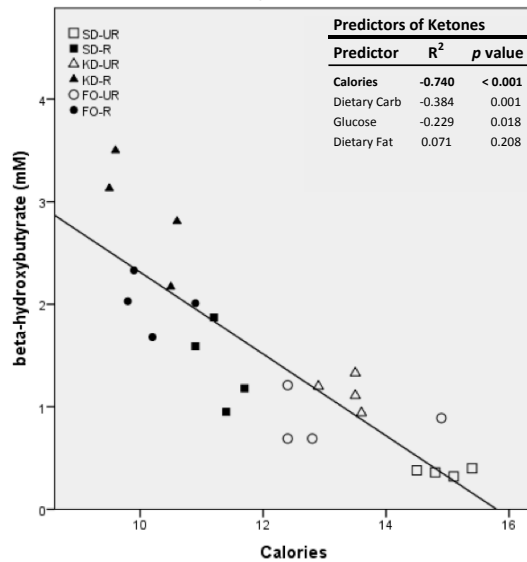
Circulating Glucose Strongly Predicts Bodyweight



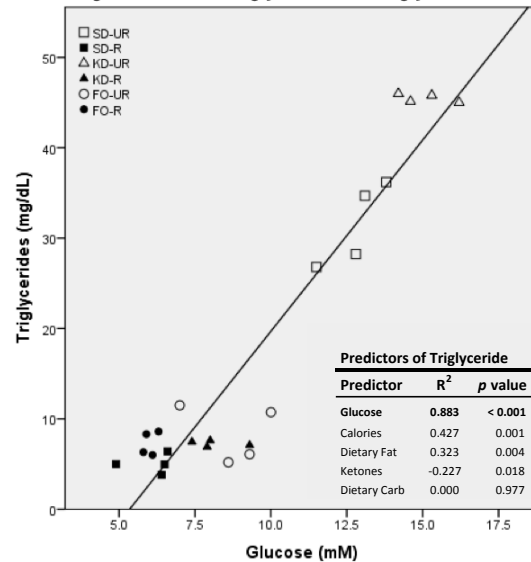
Calorie Intake Moderately Predicts Circulating Glucose



Calorie Intake Strongly Predicts Ketone Levels



Circulating Glucose Strongly Predicts Triglyceride Level





**Table 3. Composition (%) of Standard Diet, Ketogenic Diet, and Fish-Oil supplemented Diet**

<b>Components</b>	<b>Standard Diet (SD)</b>	<b>Ketogenic Diet<sup>a</sup> (KD)</b>	<b>Fish-Oil Diet<sup>b</sup> (FO)</b>
Carbohydrate	52	1	39
Fat	12	70	34
Protein	23	13	17
Fiber	4	11	3
Ash	6	3	4
Moisture	3	2	3
Metabolizable Energy (kcal/g)	3.20	6.35	4.65

<sup>a</sup> Lard-based ketogenic diet

<sup>b</sup> 1g of fish oil capsule per 3g of SD; each gram contains 180 mg EPA and 120 mg DHA

**Table 4. Influence of dietary intake on body weight and calorie consumption in C57BL/6J mice**

	Diet					
	Standard Diet		Ketogenic Diet		Fish-Oil Diet	
	UR	R	UR	R	UR	R
Final Bodyweight (g) <sup>a</sup>	29.0 ± 0.7	23.6 ± 0.1	32.5 ± 0.7	23.3 ± 0.3	27.9 ± 0.9	23.1 ± 0.2
Bodyweight Change (%)	+0.3 ± 1.8	-21.1 ± 1.4	+11.2 ± 2.3	-19.9 ± 0.6	-10.7 ± 2.7	-22.4 ± 1.1
Energy Intake (kcal) <sup>b</sup>	15.0 ± 0.4	11.3 ± 0.6	13.4 ± 0.3	10.1 ± 0.3	13.1 ± 0.6	10.2 ± 0.2
Calorie Difference (%) <sup>c</sup>	0.0 ± 1.3	-24.4 ± 1.1	0.0 ± 1.2	-24.9 ± 2.2	0.0 ± 4.6	-22.3 ± 1.9
from UR Diet						
Calorie Difference (%) <sup>d</sup>	0.0 ± 1.3	-24.4 ± 1.1	-10.5 ± 1.1	-32.8 ± 1.9	-12.2 ± 4.0	-31.8 ± 1.7
from SD-UR						

<sup>a</sup> Values are expressed as the mean ± SEM (n = 4 mice per group)

<sup>b</sup> Mean metabolizable energy (kcal) consumed per mouse per day from days 7-31

<sup>c</sup> From UR diet

<sup>d</sup> From SD-UR

**Table 5. Influence of dietary intake on plasma metabolites, hormones, and lipids in C57BL/6J mice**

	Diet					
	Standard Diet		Ketogenic Diet		Fish-Oil Diet	
	UR	R	UR	R	UR	R
<b>Metabolites<sup>a</sup></b>						
Glucose (mM)	12.8 ± 0.5	6.1 ± 0.4*	15.1 ± 0.4 <sup>§</sup>	8.5 ± 0.5* <sup>§</sup>	8.7 ± 0.6 <sup>§</sup>	6.0 ± 0.1* <sup>§</sup>
β-Hydroxybutyrate (mM)	0.4 ± 0.0	1.4 ± 0.2*	1.1 ± 0.1 <sup>§</sup>	2.9 ± 0.2* <sup>§</sup>	0.9 ± 0.1 <sup>§</sup>	2.0 ± 0.1* <sup>§</sup>
Glucose/β-Hydroxybutyrate Ratio	35.4 ± 4.7	4.6 ± 1.0*	13.4 ± 2.5 <sup>§</sup>	2.9 ± 0.9* <sup>§</sup>	10.4 ± 2.2 <sup>§</sup>	3.0 ± 0.3* <sup>§</sup>
<b>Hormones<sup>a</sup></b>						
Insulin (pmol/L)	182 ± 17	108 ± 3*	160 ± 7	110 ± 4* <sup>§</sup>	112 ± 1 <sup>§</sup>	98 ± 2 <sup>§</sup>
Adiponectin (µg/mL)	9.2 ± 0.4	18.9 ± 3.8*	11.1 ± 1.2	15.3 ± 0.8	8.7 ± 0.3	13.8 ± 1.0
<b>Lipids (mg/dL)<sup>a</sup></b>						
Triglyceride	31.5 ± 3.2	5.0 ± 0.8*	45.5 ± 0.3 <sup>§</sup>	7.3 ± 0.2* <sup>§</sup>	8.4 ± 2.3 <sup>§</sup>	7.3 ± 1.0 <sup>§</sup>
Cholesterol	28.3 ± 2.0	15.9 ± 2.8*	45.9 ± 2.6 <sup>§</sup>	30.3 ± 0.3*	22.8 ± 0.5	26.0 ± 2.8
Free fatty acid	42.7 ± 3.4	64.6 ± 4.5*	48.1 ± 3.5	57.1 ± 2.5* <sup>§</sup>	41.2 ± 2.4	44.9 ± 0.9
Cholesteryl Ester	32.1 ± 0.9	26.7 ± 0.5	55.8 ± 0.8 <sup>§</sup>	41.0 ± 1.4 <sup>§</sup>	31.9 ± 1.3	34.5 ± 3.4
Phosphatidylcholine	64.2 ± 3.8	42.2 ± 2.1*	107.9 ± 4.9 <sup>§</sup>	68.8 ± 2.9*	52.7 ± 2.3 <sup>§</sup>	59.5 ± 4.4
Lyso-phosphatidylcholine	9.3 ± 0.3	5.5 ± 0.5*	10.6 ± 0.3	6.7 ± 0.2	6.6 ± 0.6	5.7 ± 0.7
Phosphatidylethanolamine	4.0 ± 0.5	2.6 ± 0.4*	8.2 ± 0.9 <sup>§</sup>	5.6 ± 0.1 <sup>§</sup>	2.2 ± 0.4 <sup>§</sup>	3.0 ± 0.0
Sphingomyelin	6.8 ± 0.3	3.9 ± 2.0*	10.3 ± 0.9 <sup>§</sup>	10.2 ± 0.4 <sup>§</sup>	6.9 ± 0.1	8.2 ± 0.9

<sup>a</sup> Values are expressed as the mean ± SEM (n = 4 mice per group)

\* Indicates significance from unrestricted diet at  $p < 0.01$  as determined by one-way ANOVA

<sup>§</sup> Indicates significance from unrestricted standard diet at  $p < 0.01$  as determined by one-way ANOVA

**Table 6. Pearson bivariate correlations of body weight, macronutrient intake, and plasma metabolites**

	Bodyweight	Calorie Intake	Fat Intake	Carbohydrate Intake	Glucose	Ketone	Insulin	Adiponectin	Triglyceride	Cholesterol	Free Fatty Acid	Cholesteryl Ester	Phosphatidylcholine	Lyso-phosphatidylcholine	Phosphatidylethanolamine
Calorie Intake	<b>.751**</b>														
Fat Intake	<b>.494*</b>	.024													
Carbohydrate Intake	.004	<b>.496*</b>	<b>-.725**</b>												
Glucose	<b>.922**</b>	<b>.707**</b>	<b>.601**</b>	-.019											
Ketone	<b>-.610**</b>	<b>-.860**</b>	.266	<b>-.619**</b>	<b>-.478*</b>										
Insulin	<b>.677**</b>	<b>.687**</b>	.319	.202	<b>.745**</b>	<b>-.495*</b>									
Adiponectin	<b>-.640**</b>	<b>-.523**</b>	-.318	.107	<b>-.611**</b>	<b>.414*</b>	-.282								
Triglyceride	<b>.863**</b>	<b>.653**</b>	<b>.568**</b>	-.006	<b>.940**</b>	<b>-.477*</b>	<b>.694**</b>	<b>-.579**</b>							
Cholesterol	<b>.654**</b>	.262	<b>.837**</b>	<b>-.522**</b>	<b>.772**</b>	-.005	<b>.536**</b>	<b>-.469*</b>	<b>.811**</b>						
Free Fatty Acids	-.194	-.265	.099	-.144	-.168	.175	-.077	.123	-.200	-.142					
Cholesteryl Ester	<b>.560**</b>	.079	<b>.922**</b>	<b>-.673**</b>	<b>.667**</b>	.133	.372	-.396	<b>.704**</b>	<b>.944**</b>	.009				
Phosphatidylcholine	.358	.117	<b>.437*</b>	-.317	.401	.044	.306	-.400	<b>.480*</b>	<b>.674**</b>	-.164	<b>.550**</b>			
Lyso-phosphatidylcholine	<b>.843**</b>	<b>.661**</b>	<b>.588**</b>	-.020	<b>.939**</b>	<b>-.437*</b>	<b>.772**</b>	<b>-.537**</b>	<b>.957**</b>	<b>.834**</b>	-.221	<b>.706**</b>	<b>.536**</b>		
Phosphatidylethanolamine	<b>.555**</b>	.122	<b>.880**</b>	<b>-.500*</b>	<b>.690**</b>	.150	<b>.481*</b>	-.303	<b>.734**</b>	<b>.916**</b>	-.083	<b>.903**</b>	<b>.666**</b>	<b>.755**</b>	
Sphingomyelin	.250	-.087	<b>.722**</b>	<b>-.691**</b>	.405	.377	.324	-.209	.370	<b>.814**</b>	-.160	<b>.760**</b>	<b>.621**</b>	<b>.492*</b>	<b>.734**</b>

\* $p < 0.05$ , two-tailed t-test\*\* $p < 0.01$ , two-tailed t-test

## CHAPTER FOUR

### *Reduced glucose utilization underlies seizure protection with dietary therapy in epileptic EL mice*

#### INTRODUCTION

Dietary therapy, specifically the ketogenic diet, has been used to successfully manage seizures in individuals that are refractory to anti-epileptic drug (AED) therapy (Neal et al., 2008). Despite the success of the ketogenic diet for individuals who are refractory to AED therapy, many individuals on the ketogenic diet do not have their epilepsy fully controlled. Consequently, alternative dietary therapies are suggested to control seizures, including calorie restriction (Hartman et al., 2013; Kossoff et al., 2008; Yuen & Sander, 2014). Evidence from animal models demonstrates that calorie restriction is anticonvulsant and antiepileptic (Bough et al., 2003; Greene et al., 2001; Mantis et al., 2004).

Calorie restriction and ketogenic diets result in similar physiological changes, such an increase in circulating ketone bodies. Calorie restriction results in lowered circulating glucose, while the ketogenic diet may lower circulating glucose, depending on the amount of calories consumed (Mantis et al., 2004; Meidenbauer et al., 2014; Zuccoli et al., 2010). Circulating glucose levels are positively correlated with brain glucose levels (Rutter & Smales, 1976). While calorie restriction and the ketogenic diet are hypothesized to have similar mechanisms in conferring seizure

protection, preclinical evidence suggests that these mechanisms are not the same (Hartman et al., 2010). Additionally, while changes in circulating glucose and ketone bodies are associated with changes in seizure susceptibility, the influence of circulating glucose and ketone bodies on seizure susceptibility and brain metabolism is not well understood (Greene et al., 2003; Kossoff & Rho, 2009). Recent work demonstrates that the ketogenic diet reduces seizure-like activity in hippocampal neurons in rats, and this reduced excitability is reversed by the addition of glucose (Kawamura et al., 2014). Understanding the role that glucose and ketone bodies have in conferring seizure protection can aid in designing and tailoring effective dietary therapies for individual patients or lead to pharmacological therapy that mimics dietary therapy.

My goal was to evaluate the acute roles of glucose and  $\beta$ -hydroxybutyrate (the major circulating ketone body) in conferring seizure protection to the EL mouse, a model of multifactorial idiopathic generalized epilepsy (Meidenbauer et al., 2011; Suzuki, 1976; Todorova et al., 1999). Seizures in EL mice can be managed through AED and dietary therapy, which makes the EL mouse an ideal model to study the mechanisms through which seizure protection is conferred (Greene et al., 2001; Mantis et al., 2004; Nagatomo et al., 1996; Todorova et al., 2000). The Seyfried laboratory previously found that EL mice given a calorie-restricted ketogenic diet had reduced seizure susceptibility, but that subsequent acute supplementation of glucose resulted in significantly increased seizure susceptibility (Mantis et al., 2014). I confirmed these results using a calorie-restricted standard

mouse chow diet, and additionally analyzed the acute effect of lowering glucose utilization or increasing  $\beta$ -hydroxybutyrate availability on seizure susceptibility. Since calorie restriction chronically lowers glucose utilization, I simulated acute increased glucose utilization by supplementing glucose in the drinking water of calorie-restricted mice immediately before seizure testing. I simulated acute low glucose utilization conditions by using acute supplementation of the glycolytic inhibitor 2-deoxy-D-glucose (2-DG), a proconvulsant and anticonvulsant compound (Gasior et al., 2010). Additionally, I supplemented  $\beta$ -hydroxybutyrate in the drinking water of the mice to simulate increased ketone body availability. I found that in conjunction with long-term calorie restriction (>4 weeks), seizure protection in the EL mouse was linked to reduction of glucose utilization. Acute supplementation of  $\beta$ -hydroxybutyrate, in conjunction with long-term calorie restriction, did not increase seizure protection in the EL mouse. In the absence of long-term calorie restriction, neither acutely lowering glucose utilization nor supplementing  $\beta$ -hydroxybutyrate conferred seizure protection.

## MATERIALS AND METHODS

### Mice

The inbred, epilepsy-prone EL mice were originally obtained from J. Suzuki (Tokyo Institute of Psychiatry) and maintained in the Boston College Animal Care Facility. The mice were group housed (2-4 mice per cage) and kept on a 12-hour

light/dark cycle with lights on at 6 am and lights off at 6 pm. All mice were tested at 10 am and fed at 11 am. Mice were assigned cage mates by matching body weights during the pretrial period to minimize calorie intake differences. Mice had free access to weighed food, measured water, and their cage mates throughout the study. Females were used for these studies, as adult males die sporadically with age from acute uremia poisoning due to urinary blockage and retention (Todorova et al., 2003). Seizures in EL mice commence with sexual maturity at approximately 60-75 days of age (Greene et al., 2001; Todorova et al., 1999). One cohort of mice was used for all analyses. The procedures for animal use were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Boston College Institutional Animal Care and Use Committee.

#### Seizure Susceptibility, Seizure Testing, and Seizure Phenotype

The seizure susceptibility paradigm for testing EL mice, along with the seizure phenotypes, has been previously described (Mantis et al., 2004; Todorova et al., 1999). Briefly, mice were tested for seizures once per week through a tail suspension paradigm. Mice were characterized as seizure-susceptible if they experienced a generalized seizure. A generalized seizure in the EL mouse, which has been electroencephalographically validated, is characterized by loss of consciousness, loss of postural equilibrium, and head and limb clonus, which may also be accompanied by vocalization and incontinence (Greene et al., 2001; Suzuki, 1976; Todorova et al., 1999). An erect-forward arching Straub tail, which indicates



spinal cord activation, is also observed in EL mice having generalized seizures. EL mice that displayed vocalization and twitching without full progression to a generalized seizure were designated as not seizure susceptible (Todorova et al., 1999). Mice experiencing a generalized seizure during the weekly testing paradigm were assigned a seizure susceptibility score of 1, whereas mice not experiencing a generalized seizure were assigned a seizure susceptibility score of 0. The seizure susceptibility score for each mouse was averaged over multiple tests and the mean seizure susceptibility score for each group was determined.

#### Diet

All mice were fed with standard rodent chow *ad libitum* during the pretrial period (Prolab RMH 3000; PMI LabDiet, Richmond, IN, USA). This is the standard mouse pellet diet, which contains 4.2 kcal/g of total energy. According to manufacturer specifications, this diet is composed of 520 g of carbohydrates, 120 g of fat, 225 g of protein, and 45 g of fiber per 1 kg of food. During the study phase, SD-UR mice received rodent chow *ad libitum*, whereas all SD-R groups were calorie restricted on rodent chow to reduce mouse body weights by 20-23%. Food was weighed and administered daily. Water was provided *ad libitum* to all mice throughout the study. Food and water intake was tracked for each cage and averaged to calculate individual intake. During the study phase, 2.5 hours prior to seizure testing, water bottles in each cage were changed to contain a measured amount of water and either water alone, water plus 25 mM D-glucose (Sigma

Aldrich, St. Louis, MO), water plus 25 mM D-glucose and 8 mM 2-deoxy-D-glucose (2-DG) (Sigma Aldrich), or water plus 25 mM D-glucose and 50 mM  $\beta$ -hydroxybutyrate (bOHB) (Sigma Aldrich). Immediately before seizure testing, all water bottles were changed back to water alone.

### Pretrial Period

The pretrial period began when the mice were  $40 \pm 1$  days of age and lasted for seven weeks. During this time, all mice were group-housed and fed *ad libitum*. They were tested for seizure susceptibility once per week. Only mice that had demonstrated at least one generalized seizure during the pretrial period were included in the study. The mean number of seizures that mice experienced before beginning the study was  $2.57 \pm 0.13$  (standard error of the mean).

### Treatment Period

After the 7-week pretrial period, the mice were arranged into five groups ( $n = 13-15$  mice/group). All mice were then fasted for 16 hours to establish a similar metabolic state at the start of the experiment. The mice in each group were assigned to one of five dietary conditions:

- 1) Standard diet (SD) fed *ad libitum* with water alone prior to seizure testing (SD-UR),
- 2) SD restricted to achieve a 20-23% body weight reduction with water alone prior to seizure testing (SD-R),

- 3) SD restricted to achieve a 20-23% body weight reduction with water plus 25 mM glucose prior to seizure testing (SD-R[Glu]),
- 4) SD restricted to achieve a 20-23% body weight reduction with water plus 25 mM glucose and 8 mM 2-DG prior to seizure testing (SD-R[Glu][2-DG]), and
- 5) SD restricted to achieve a 20-23% body weight reduction with water plus 25 mM glucose and 50 mM bOHB prior to seizure testing (SD-R[Glu][bOHB]).

The treatment period lasted for a total of 10 weeks, and included weekly seizure testing through 9 weeks.

#### Collection of Plasma and Brain

Blood was collected at the end of the pretrial phase (1 hour after seizure testing) and on week 9 (1 hour after seizure testing) through submandibular bleeding into heparinized tubes. Plasma was collected by centrifuging blood at 3,000 x *g* for 10 minutes at 4°C and stored at -80°C until analysis. Whole brain was collected one week after the end of seizure testing (week 10) through snap freezing in liquid nitrogen. The whole brain collection procedure occurred immediately after a 2.5-hour administration of water from each respective group to mimic the metabolic state of the mouse during the seizure-testing paradigm.

### Measurement of Plasma Glucose and $\beta$ -hydroxybutyrate

Glucose was measured spectrophotometrically using the Trinder Assay (StanBio, Boerne, TX).  $\beta$ -hydroxybutyrate was measured enzymatically using a modification of the Williamson *et al.* procedure (Williamson et al., 1962).

### Measurement of Brain Hexokinase Activity

Hexokinase activity was measured from the cerebrum using a hexokinase colorimetric assay kit (Sigma Aldrich). Hexokinase activity was standardized to protein levels using the Bio-Rad protein assay (Hercules, CA).

### Metabolite Extraction and $^1\text{H}$ -NMR Analysis

Whole brain samples were extracted with ice-cold 2:1 methanol:chloroform. Samples were centrifuged at  $8,000 \times g$  for 20 minutes at  $4^\circ\text{C}$  and the supernatant was collected. Extraction was performed one more time on the pellets, and the supernatants were pooled. Samples were dried down under a stream of nitrogen and lyophilized. The samples were rehydrated in deuterium oxide ( $\text{D}_2\text{O}$ ) for  $^1\text{H}$  NMR analysis.

$^1\text{H}$  NMR spectra of the extracts were obtained using a Varian VNMRs 600 spectrometer at 599.688 MHz at  $25^\circ\text{C}$  using a 9615.4 Hz sweep width, 32,768 data points collected, and a 1.0 second relaxation delay. Sixty-four transients were collected and the resulting free induction decay was processed with a line broadening of 0.5 Hz. Metabolite ratios for lactate, *N*-acetylaspartate (NAA),  $\gamma$ -

aminobutyric acid (GABA), glutamate, aspartate, and taurine were calculated by integrating the selected reference peaks (**Figure 11**) (Govindaraju et al., 2000). The area under the curve for each reference peak was standardized to the area under the curve for phosphocreatine plus creatine.

#### Measurement of Glutamate Decarboxylase Activity

Glutamate decarboxylase (GAD) activity was measured fluorescently with an NADPH-coupled assay, according to the methods of Wolf and Klemisch (Wolf & Klemisch, 1991). All reagents for this assay were obtained from Sigma Aldrich. Briefly, frozen whole brain tissue was homogenized in buffer (0.1 M sodium phosphate buffer with 1 mM 2-aminoethylisothiuronium bromide, 0.1% Triton X-100, and 20  $\mu$ M pyridoxal 5'-phosphate; pH 7.0) and centrifuged at 5,000  $\times g$  for 30 minutes at 4°C. The supernatant was collected and mixed with buffer containing glutamate (0.1 M sodium phosphate buffer with 50 mM L-glutamate, 250  $\mu$ M pyridoxal 5'-phosphate, and 0.4% beta-mercaptoethanol; pH 7.0) to drive the GAD reaction for 15 minutes at 37°C. The reaction was stopped with the addition of 0.25 N HCl. Samples were placed in a dry bath at 100°C for 5 minutes to destroy endogenous NADPH. Samples were mixed in a buffer with GABase (0.3 M Tris buffer with 6 mM alpha-ketoglutarate, 0.1 M NADP<sup>+</sup>, 6 mM beta-mercaptoethanol, and 10 U GABase; pH 8.4) for 15 minutes at 37°C to drive the indicator reaction to completion. NADPH is formed in an equimolar ratio with GABA formation, and

NADPH was measured fluorescently with an excitation wavelength of 340 nm and an emission wavelength of 460 nm.

### Statistical Analyses

All statistical analyses were performed using SPSS Software (IBM SPSS Statistics, Version 21). One-way ANOVA was used to evaluate any significant differences in body weight, seizure susceptibility, plasma glucose, plasma  $\beta$ -hydroxybutyrate levels, brain metabolites, and GAD activity between treatment groups. Differences were considered significant at  $p < 0.05$ . All values are presented as mean  $\pm$  standard error of the mean (SEM).

## RESULTS

### Influence of Treatments on Body Weight

The average body weight at the start of the treatment period was  $27.7 \pm 0.3$  g. The treatments, with the exception of SD-UR, lowered body weights by 20-23% and did not lead to any statistical difference in body weights for the calorie restricted groups (**Figure 12**).

### Water Intake and Drug Dosages

Water intake during the 2.5-hour treatment period was similar across all groups ( $1.59 \pm 0.07$  mL). The mean glucose intake in the groups receiving glucose

was similar at  $7.62 \pm 0.52$  mg. This was a dose of 375 mg/kg for glucose. The mean 2-DG intake was  $2.01 \pm 0.19$  mg for the SD-R[Glu][2DG] group, and the mean bOHB intake was  $9.52 \pm 0.07$  mg for the SD-R[Glu][bOHB] group. The doses for 2-DG and bOHB were 100 mg/kg and 475 mg/kg, respectively. The addition of 2-DG decreased brain hexokinase activity in the SD-R[Glu][2-DG] group ( $3.5 \pm 1.2$  nM/min/mg protein) compared with all other groups combined ( $12.5 \pm 2.5$  nM/min/mg protein).

#### Influence of Treatments on Seizure Susceptibility

In the absence of long-term calorie restriction, the acute treatments of glucose, glucose plus 2-DG, and glucose plus bOHB did not affect seizure susceptibility (**Figure 13**). With long-term calorie restriction, however, treatment differences were observed (**Figure 14**). The SD-UR group displayed high seizure susceptibility, which was reduced through calorie restriction, as seen in the SD-R group. The addition of glucose to the drinking water of the mice for 2.5 hours prior to seizure testing increased seizure susceptibility. The increase in seizure susceptibility was blocked by the addition of 2-DG to the drinking water. Adding 50 mM bOHB to the drinking water, which was the highest concentration of bOHB in water that EL mice could tolerate before restricting water intake, did not ameliorate the effects of 25 mM glucose on seizure susceptibility.

### Influence of Treatments on Plasma Glucose and $\beta$ -hydroxybutyrate Levels

At the beginning of the study, all mice had similar plasma glucose ( $10.8 \pm 0.1$  mM) and bOHB levels ( $0.40 \pm 0.02$  mM). The plasma glucose levels after 9 weeks of treatment were significantly lower in all SD-R groups, compared with the SD-UR (**Figure 15**). Plasma bOHB levels were significantly higher in all SD-R groups, compared with the SD-UR group (**Figure 16**). The addition of glucose, 2-DG, and bOHB to the drinking water did not alter plasma glucose and bOHB levels across SD-R groups.

### Influence of Treatments on Brain Metabolites

The brain metabolites of lactate, NAA, GABA, glutamate, aspartate, and taurine were measured using  $^1\text{H}$  NMR spectroscopy. The excitatory amino acids, glutamate and aspartate, along with the inhibitory amino acids, GABA and taurine, were unchanged in the brain of all dietary groups (**Table 7**). *N*-acetylaspartate, a neuronal marker of pathology, was also unchanged in all groups. Lactate, a neuronal energy substrate, was additionally unchanged in all groups.

### Influence of Treatments on Brain GAD Activity

Brain GAD activity was significantly increased across all calorie-restricted groups compared with the SD-UR group (**Figure 17**). Glutamate decarboxylase activity was similar across all calorie-restricted groups, indicating that acute treatments of glucose, 2-DG, and bOHB did not affect GAD activity.



## DISCUSSION

I confirmed previous work that long-term calorie restriction significantly reduces seizure susceptibility in EL mice (Mantis et al., 2004), and that acute addition of glucose abolishes the seizure-protective effects of calorie restriction (Mantis et al., 2014). I found that reduced glucose utilization is necessary for acutely managing seizures with dietary therapy in EL mice, whereas acute supplementation of bOHB does not have a seizure-protective role for EL mice, in the presence of increased glucose availability. Interestingly, in the absence of long-term calorie restriction, the acute reduction of glucose utilization did not decrease seizure susceptibility.

The induction of ketosis is a common motif among dietary therapies that are used to treat epilepsy. Our data indicate that acute supplementation of bOHB was unable to confer seizure protection in EL mice. The ketone body bOHB, which is the major circulating ketone body, has not demonstrated acute anticonvulsant activity in a variety of models, whereas the ketone body acetoacetate has been shown to have anticonvulsant activity (Kossoff & Rho, 2009). We administered bOHB in the drinking water, as the EL mice did not restrict water intake. Additionally,  $\beta$ -hydroxybutyrate dehydrogenase in the liver readily interconverts bOHB and acetoacetate to maintain a circulating equilibrium, and the 2.5-hour treatment period would allow ample time for the equilibrium to be maintained (Veech, 2004; Williamson et al., 1962).

These results, therefore, suggest that acute ketone body supplementation does not confer seizure protection in EL mice. This is consistent with previous results demonstrating that chronic bOHB supplementation in the drinking water of calorie-restricted EL mice did not result in additional anticonvulsant activity compared with calorie-restricted controls (Mantis et al., 2014). I cannot completely rule out that supplementing higher levels of ketone bodies may be anticonvulsant in the EL mouse; however, a solution of 50 mM bOHB was the highest concentration EL mice could tolerate before restricting fluid intake. Furthermore, plasma levels of bOHB were not increased with bOHB supplementation, which suggests rapid utilization or blood clearance of bOHB. While bOHB may not be readily utilized for energy in the initial stages of calorie restriction, supplemented bOHB should be readily metabolized with long-term calorie restriction as brain cells upregulate activity of enzymes important in ketone body metabolism, although this did not result in protection against seizures (Greene et al., 2003; Mantis et al., 2004; Veech, 2004).

In contrast to ketone supplementation, acutely decreasing glucose utilization resulted in seizure protection for EL mice under long-term calorie restriction. Decreased glucose utilization was achieved with administration of 2-DG, a glycolytic inhibitor that produces the metabolite 2-deoxy-D-glucose-6-phosphate, which allosterically inhibits the phosphorylating action of hexokinase, a rate-limiting step of glycolysis (Chen & Gueron, 1992; Horton et al., 1973). 2-DG is readily taken up into the brain of EL mice, and we confirmed that 2-DG administration reduced

hexokinase activity in the brain of EL mice (Nakamoto et al., 1990). Acute supplementation of 2-DG did not affect circulating glucose levels. While decreasing glucose utilization conferred seizure protection in mice under long-term calorie restriction, it did not confer seizure protection with short-term calorie restriction. This suggests that the mechanisms for seizure control from a calorie-restricted diet are multifaceted.

The circulating levels of glucose and ketones were not associated with changes in seizure protection due to acute administration of glucose, 2-DG, or bOHB, as circulating glucose and ketones did not change with acute treatment under long-term calorie restriction. This is consistent with previous work demonstrating that acute supplementation of glucose does not lead to an increase in circulating glucose levels (Mantis et al., 2014). This is also consistent with other work demonstrating that low circulating blood glucose leads to rapid blood glucose clearance after a bolus of glucose (Verdonk et al., 1981), and ketone bodies are rapidly cleared from plasma in rats under calorie-restricted conditions (Hawkins et al., 1986). Rapid blood glucose clearance is associated with increased cerebral glucose utilization and seizures (Cornford et al., 2000; Mantis et al., 2014). Rapid bOHB clearance does not appear to be associated with seizure incidence.

Seizure susceptibility can be acutely changed with the addition of glucose or 2-DG; I, therefore, examined the hypothesis that calorie restriction and ketosis lead to mitochondrial flux and change the brain handling of amino acids, particularly the conversion of glutamate to GABA (Yudkoff et al., 2007). A previous report on EL

mice found that the mice have enhanced release of calcium-dependent potassium-evoked aspartate release in hippocampal neurons, but no changes in glutamate or GABA release (Flavin et al., 1991; Flavin & Seyfried, 1994). I found no changes in whole brain amino acid levels. A previous report examining seizures in rat pups had reported no changes in brain GABA levels related to seizure activity but had reported changes in GAD levels related to seizure activity (Arias et al., 1992). This suggested that the metabolic flux of glutamate decarboxylation to GABA is more representative of changes in seizure-related brain metabolism than steady-state levels of brain amino acids. We found that GAD activity was increased across all calorie-restricted groups compared with the SD-UR group but was similar across all calorie-restricted groups, regardless of seizure susceptibility. This demonstrated that increased GAD activity was not associated with acute seizure protection. Increased GAD activity may be necessary under long-term calorie restriction to confer seizure protection, but other processes must also be responsible for conferring acute seizure protection. These data also do not rule out that increased GAD activity may be a side effect of calorie restriction that is unrelated to seizure protection. This result underscores the utility of the present testing paradigm in parsing the effects of calorie restriction from seizure protection, thereby revealing the effects of calorie restriction that are dissociated from seizure protection, such as increased GAD activity.

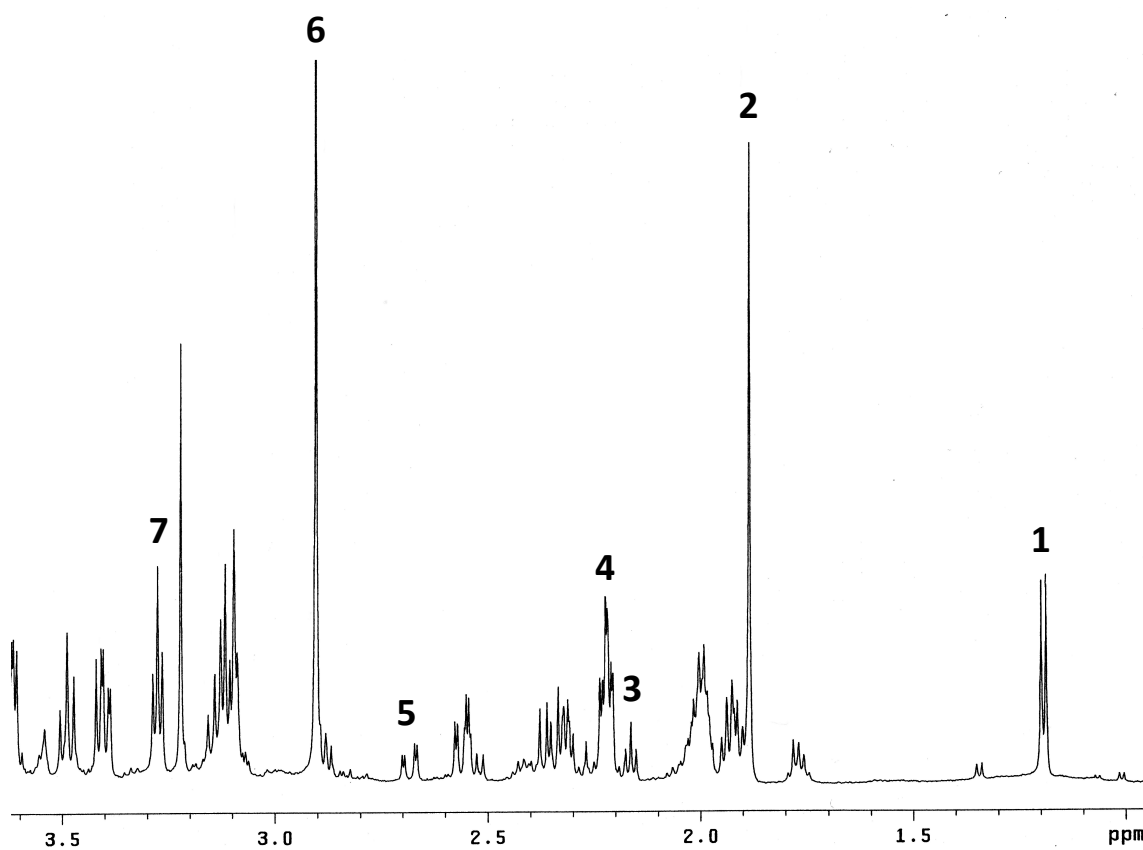
A possible mechanism that would confer both chronic seizure protection and acute seizure protection in EL mice that remains consistent with my present data is

the hypothesis that metabolism of ketones raises the free energy of ATP hydrolysis, which leads to an increase in the resting membrane potential of neurons, thereby inhibiting aberrant synchronous neuronal discharges (**Figure 18**) (Sato et al., 1995; Veech et al., 2001; Veech et al., 2002; Veech, 2004). Efficient brain ketone metabolism requires upregulating monocarboxylic acid transporters and relevant ketone body metabolizing enzymes, which occurs after prolonged fasting, calorie restriction or a ketogenic diet (Maalouf et al., 2009; Morris, 2005). Therefore, providing exogenous ketones or acutely decreasing glucose utilization would not confer seizure protection until ketones can be efficiently metabolized to increase the resting membrane potential of neurons. During prolonged fasting or calorie restriction, when glucose becomes available, neurons will utilize glucose through glycolysis, as glycolysis produces ATP faster than the oxidative steps of the tricarboxylic acid cycle and the electron transport chain (Greene et al., 2003; McIlwain & Tresize, 1956). This acute glucose utilization would lead to neuronal depolarization, which could increase aberrant synchronous neuronal discharges and allow breakthrough seizures (Huttenlocher, 1976; Mantis et al., 2014). The present and previous results with dietary therapy in EL mice remain consistent with the hypothesis of ketone body metabolism leading to neuronal hyperpolarization (Mantis et al., 2014).

I demonstrated that under long-term calorie restriction, decreasing glucose utilization is necessary for seizure protection. Ketone body supplementation did not provide acute seizure protection to EL mice with or without long-term calorie

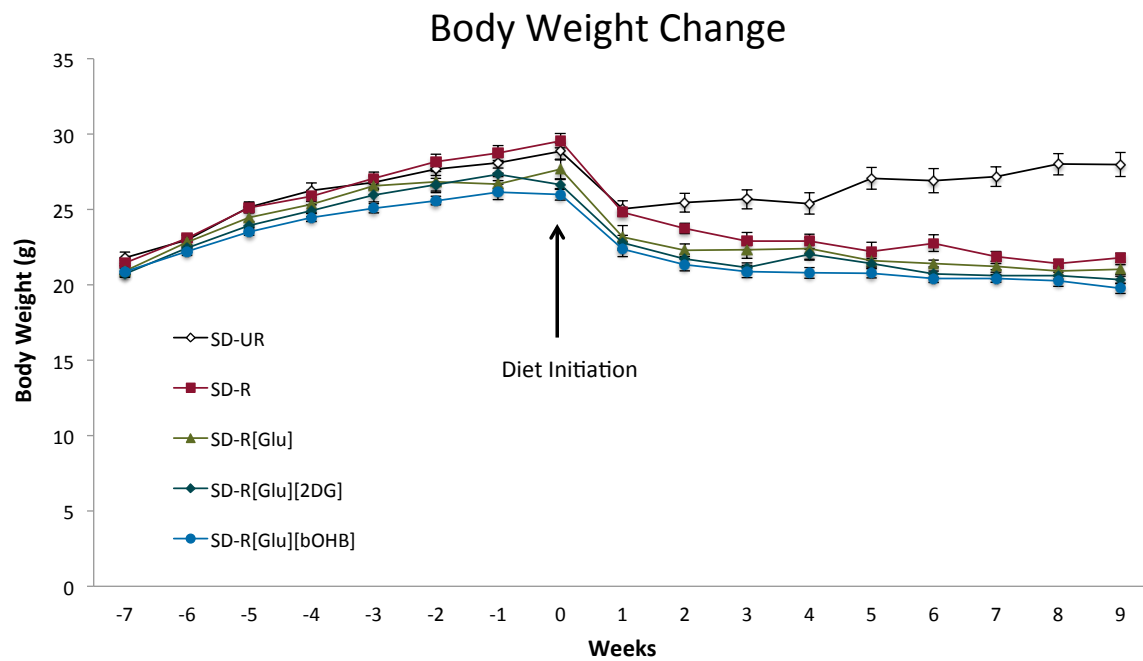
restriction. In the absence of long-term calorie restriction, decreased glucose utilization did not provide seizure protection to the EL mouse.

**Figure 11.** Representative  $^1\text{H}$  NMR spectra from an EL mouse brain obtained at 600 MHz. Reference peaks correspond to the following metabolites: 1, lactate; 2, *N*-acetylaspartate (NAA); 3,  $\gamma$ -aminobutyric acid (GABA); 4, glutamate; 5, aspartate; 6, phosphocreatine plus creatine; and 7, taurine (Govindaraju et al., 2000).



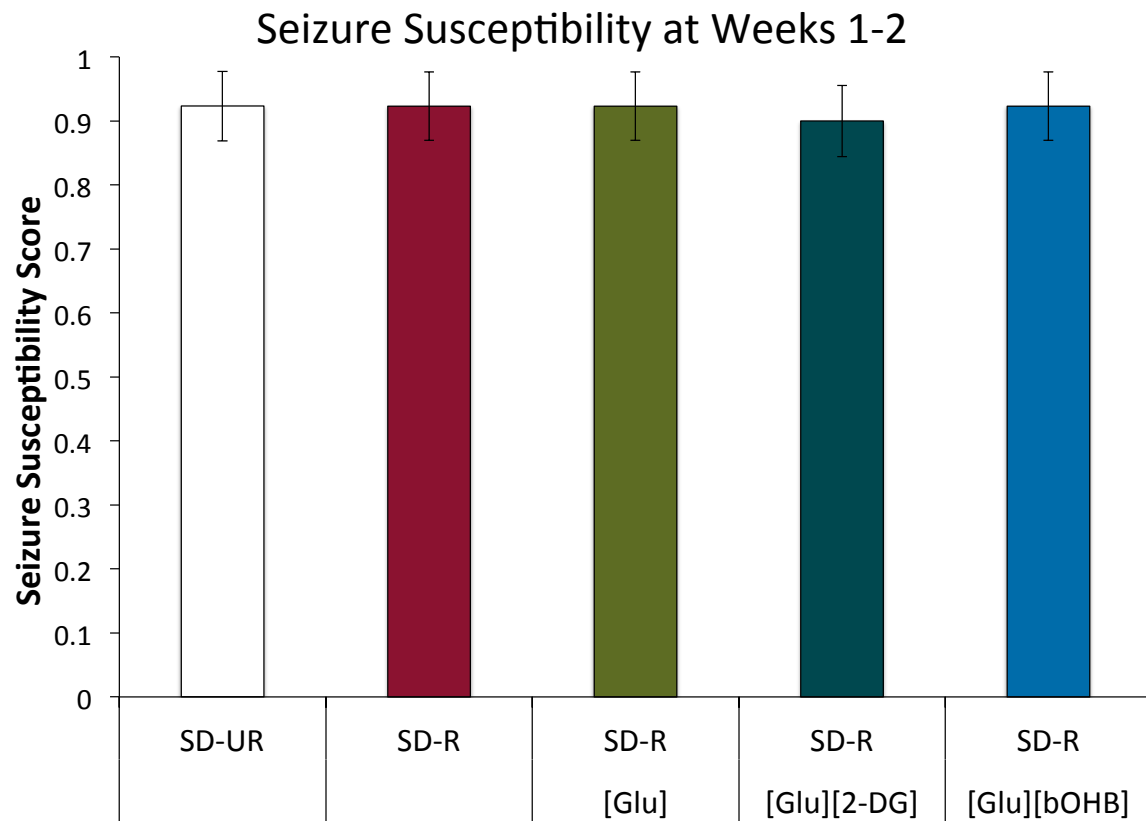


**Figure 12.** Effect of dietary treatment on body weight. At week 0, dietary treatment was initiated. From week 2 to week 9, body weights in the SD-UR group were significantly higher than those in all SD-R groups ( $p < 0.01$ ). Values are displayed as mean  $\pm$  SEM ( $n = 13-15$  mice/group).



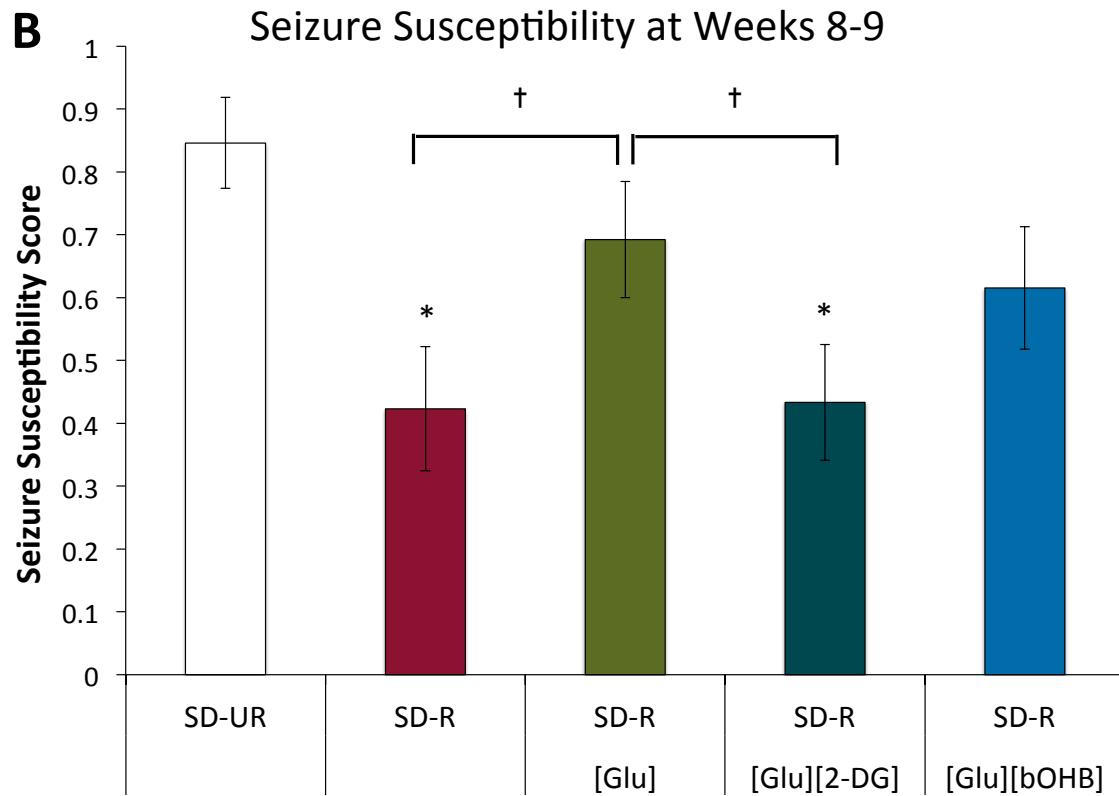
**Figure 13.** Seizure susceptibility in EL mice with dietary treatment for weeks 1-2.

Seizure susceptibility scores were pooled for weeks 1-2 to assess the effect of acute glucose, glucose plus 2-DG, and glucose plus bOHB treatment in the absence of long-term calorie restriction. Seizure susceptibility was similar across all groups.

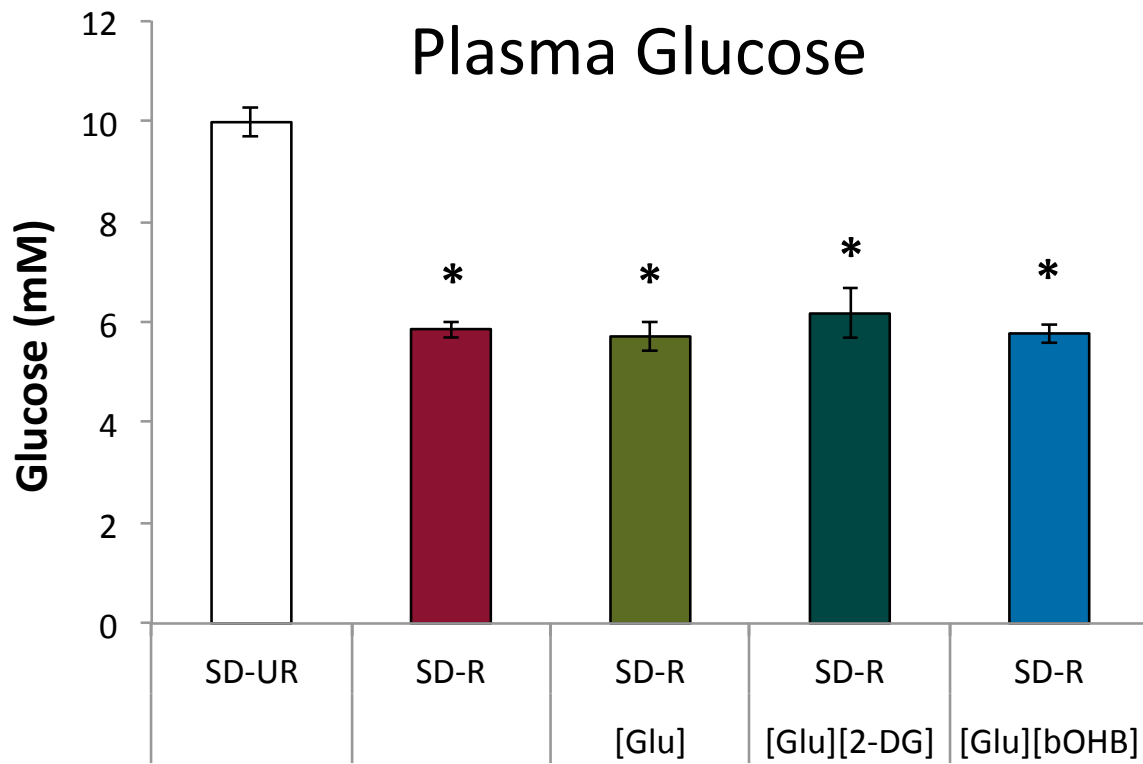


**Figure 14.** Seizure susceptibility in EL mice with dietary treatments for weeks 8-9.

Seizure susceptibility scores were pooled for weeks 8-9, as the seizure susceptibilities were consistent within groups at the study end-point. Seizure susceptibility was reduced in the SD-R group, and the effect of calorie restriction was blocked by the addition of 25 mM D-glucose in the drinking water prior to testing (SD-R[Glu]). The addition of 8 mM 2-DG to the 25 mM D-glucose in the drinking water (SD-R[Glu][2-DG]) significantly lowered seizure susceptibility and returned seizure susceptibility levels to that of SD-R. 50 mM bOHB did not ameliorate the effect of glucose on seizure susceptibility (SD-R[Glu][bOHB]). Values are displayed as mean  $\pm$  SEM ( $n = 13-15$  mice/group). \* $p < 0.01$  compared with the SD-UR group; † $p < 0.05$ .

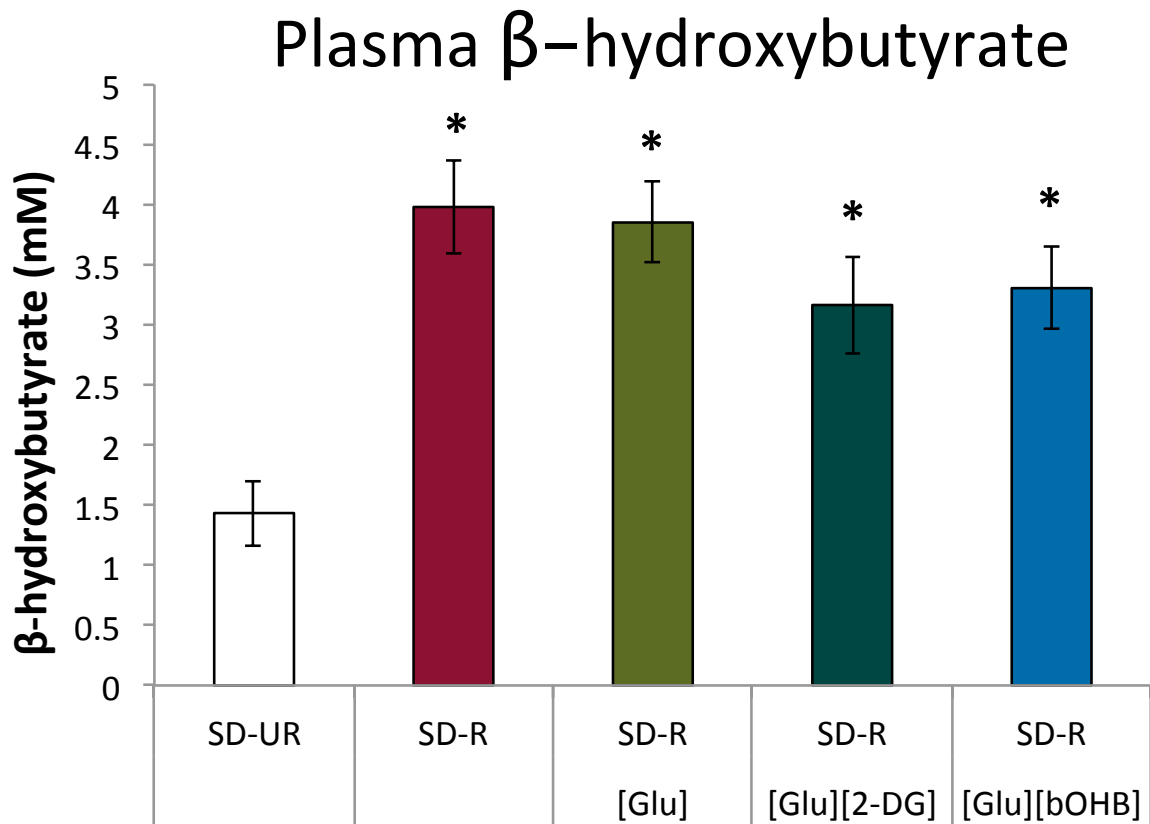


**Figure 15.** Influence of dietary treatment on plasma glucose levels in EL mice after 9 weeks of treatment. Plasma glucose levels were significantly reduced in all of the SD-R groups. Values are displayed as mean  $\pm$  SEM ( $n = 13-15$  mice/group).  $*p < 0.01$ .



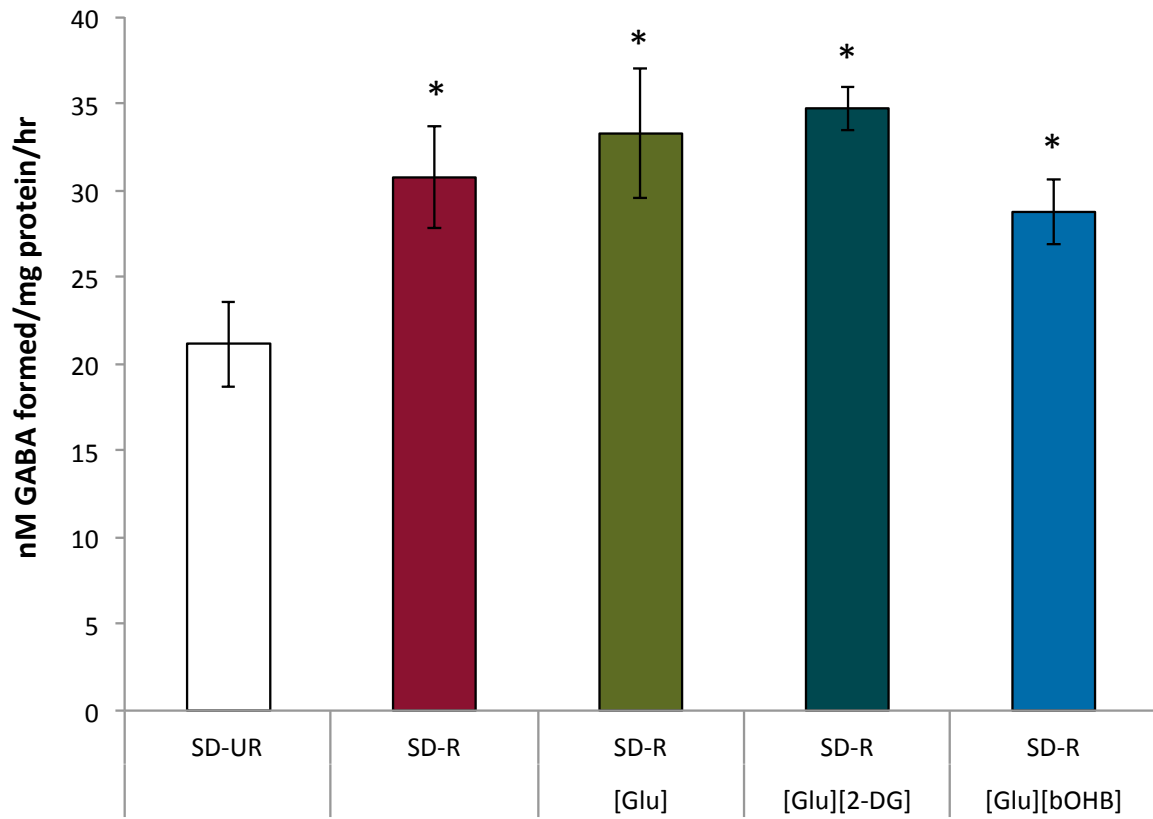


**Figure 16.** Influence of dietary treatment on plasma  $\beta$ -hydroxybutyrate levels in EL mice after 9 weeks of treatment. Plasma  $\beta$ -hydroxybutyrate levels were significantly raised in all of the SD-R groups. Values are displayed as mean  $\pm$  SEM ( $n$  = 13-15 mice/group). \* $p$  < 0.01.



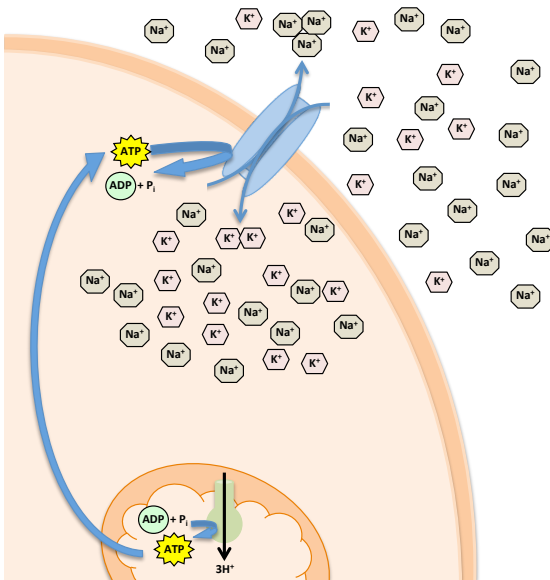
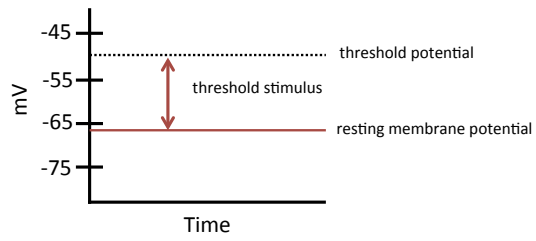
**Figure 17.** Effect of dietary treatment on brain glutamate decarboxylase (GAD) activity. GAD activity is significantly increased in all calorie-restricted groups, compared with the SD-UR group. GAD activity is similar across all calorie-restricted groups. Values are displayed as mean  $\pm$  SEM ( $n = 5-6$  mice/group).  $*p < 0.05$  compared with the SD-UR group.

## Brain Glutamate Decarboxylase Activity

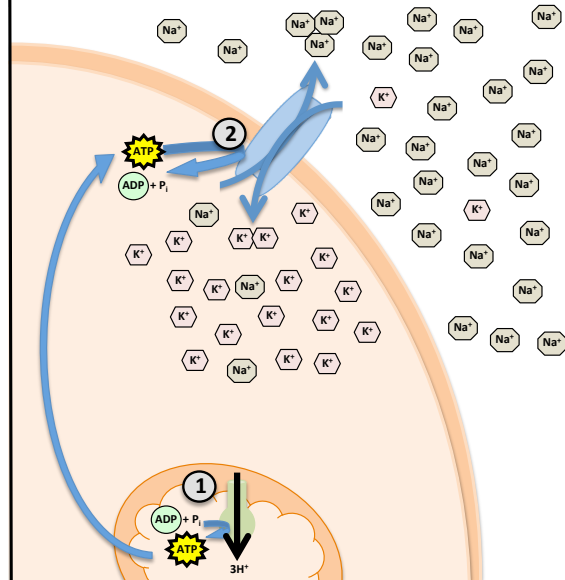
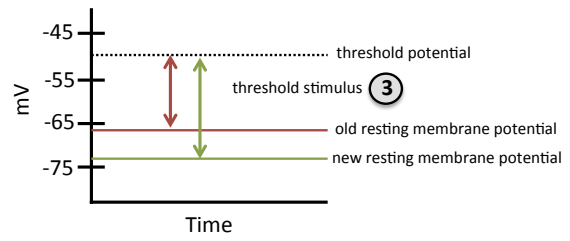


**Figure 18.** Ketone utilization hyperpolarizes the resting membrane potential of neurons. **(1)** Ketone utilization increases the mitochondrial redox span, leading to an increase in the free energy of ATP hydrolysis (see Sato *et al.*, 1995). As a result of increasing the free energy of ATP hydrolysis, **(2)** Na<sup>+</sup>/K<sup>+</sup> pumps increase the Na<sup>+</sup> and K<sup>+</sup> gradient, leading to high intracellular K<sup>+</sup> and high extracellular Na<sup>+</sup>. **(3)** The new resting membrane potential is hyperpolarized, which requires a larger threshold stimulus for neuronal firing.

## Glucose Utilization



## Ketone Utilization



**Table 7. Effect of diet and treatment on brain metabolites**

Treatment Group	Metabolite*					
	Lactate	N-acetylaspartate	GABA	Glutamate	Aspartate	Taurine
SD-UR	40.8 ± 2.1	57.0 ± 6.5	16.6 ± 2.2	75.6 ± 2.2	18.1 ± 1.9	57.9 ± 3.9
SD-R	36.6 ± 1.4	56.3 ± 3.9	12.7 ± 0.7	73.1 ± 2.9	15.3 ± 0.8	56.5 ± 3.0
SD-R[Glu]	39.1 ± 1.9	55.8 ± 1.9	10.9 ± 0.7	70.9 ± 4.1	13.4 ± 1.3	53.1 ± 2.8
SD-R[Glu][2DG]	40.5 ± 2.6	57.0 ± 2.9	10.2 ± 1.4	74.8 ± 1.8	13.3 ± 1.9	53.2 ± 2.5
SD-R[Glu][bOHB]	34.4 ± 4.0	57.2 ± 2.7	11.9 ± 0.7	72.9 ± 2.0	15.0 ± 0.2	57.2 ± 2.2

\*Values are ratios of <sup>1</sup>H NMR peaks standardized to <sup>1</sup>H NMR phosphocreatine + creatine peaks, represented as mean ± SEM. *n* = 4-6 mice per group.

## CHAPTER FIVE

*The Glucose Ketone Index Calculator: a simple tool to help manage malignant brain cancer.*

### INTRODUCTION

Dietary therapy using ketogenic diets is emerging as an alternative or complementary approach to the current standard of care for brain cancer management. Prognosis remains poor for malignant gliomas in both children and adults (Armstrong et al., 2006; Artico et al., 1993; Fisher & Buffler, 2005; Harbaugh & Black, 1998; Seyfried et al., 2014b). Although genetic heterogeneity is extensive in malignant gliomas (Brennan et al., 2013; Johnson et al., 2014; Patel et al., 2014), the Warburg effect (aerobic fermentation of glucose) is a common metabolic malady expressed in nearly all neoplastic cells of these and other malignant tumors (Ferreira, 2010; Seyfried & Mukherjee, 2005b; Seyfried et al., 2014a; Seyfried et al., 2014b). Aerobic fermentation is necessary to compensate for the insufficiency of mitochondrial oxidative phosphorylation in the cells of most tumors (Ferreira, 2010; Seyfried et al., 2014a; Warburg, 1956a; Warburg, 1956b). Mitochondrial structure and function is abnormal in malignant gliomas from both mice and humans (Arismendi-Morillo & Castellano-Ramirez, 2008; Deighton et al., 2014a; Deighton et al., 2014b; Kiebish et al., 2008; Oudard et al., 1997; Scheithauer & Bruner, 1987; Seyfried & Mukherjee, 2005b; Seyfried et al., 2011; Sipe et al., 1973).



Normal brain cells gradually transition from the metabolism of glucose to the metabolism of ketone bodies (primarily  $\beta$ -hydroxybutyrate and acetoacetate) for energy when circulating glucose levels become limiting (Cahill & Veech, 2003; Cahill, 2006; Krebs et al., 1971). Ketone bodies are derived from fatty acids in the liver and are produced to compensate for glucose depletion during periods of food restriction (Cahill & Veech, 2003). Ketone bodies bypass the glycolytic pathway in the cytoplasm and are metabolized directly to acetyl CoA in the mitochondria (Seyfried et al., 2005; VanItallie & Nufert, 2003; Veech et al., 2001). Tumor cells are less capable than normal cells in metabolizing ketone bodies for energy due to their mitochondrial defects (Seyfried et al., 2014a; Seyfried et al., 2014b).

Therapies that can lower glucose and elevate ketone bodies will place more energy stress on the tumor cells than on the normal brain cells (Klement & Kammerer, 2011; Seyfried et al., 2014a). This therapeutic strategy is illustrated conceptually in **Figure 19**, as has been previously described (Abdelwahab et al., 2012; Seyfried & Mukherjee, 2005b; Seyfried et al., 2008). However, daily activities and emotional stress can cause blood glucose levels to vary, which makes it difficult for some individuals to enter the predicted therapeutic zone of tumor management (Goetsch et al., 1990). A more stable measure of systemic energy metabolism is therefore needed to predict metabolic management of tumor growth. The ratio of blood glucose to blood ketone bodies  $\beta$ -hydroxybutyrate (bOHB) is a clinical biomarker that could provide a better indication of metabolic management than could measurement of either blood glucose or ketone body levels alone.

## MATERIALS AND METHODS

The 'Glucose Ketone Index' (GKI) was created to track the metabolic zone of brain tumor management. The GKI is a biomarker that refers to the molar ratio of circulating glucose over bOHB, which is the major circulating ketone body. A mathematical tool called the Glucose Ketone Index Calculator (**Supporting File 1**) was developed that can calculate the GKI and monitor changes in this parameter on a daily basis (**Equation 1**). The GKIC generates a single value that can assess the relationship of the major fermentable tumor fuel (glucose) to the non-fermentable fuel (bOHB). Because many commercial blood glucose monitors give outputs in mg/dL, rather than millimolar (mM), the GKIC converts the units to millimolar. Included in the program is a unit converter for both glucose and ketones (bOHB), which can convert glucose and ketone values from mg/dL to mM and from mM to mg/dL (**Figure 20**) (**Equations 2-5**). The molecular weights used for calculations in the GKIC are 180.16 g/mol for glucose and 104.1 g/mol for bOHB, the major circulating ketone body that is measured in most commercial testing kits. The unit converter allows for compatibility for a variety of glucose and ketone testing monitors.

$$(1) \quad [\text{Glucose Ketone Index}] = \frac{[\text{Glucose (mg/dL)}]/18.016}{[\text{Ketone (mM)}]}$$

$$(2) \quad [\text{Glucose (mg/dL)}] = [\text{Glucose (mM)}] \times 18.016$$

$$(3) \quad [\text{Glucose (mM)}] = \frac{[\text{Glucose (mg/dL)}]}{18.016}$$

$$(4) \quad [\text{Ketone (mg/dL)}] = [\text{Ketone (mM)}] \times 10.41$$

$$(5) \quad [\text{Ketone (mM)}] = \frac{[\text{Ketone (mg/dL)}]}{10.41}$$

The GKIC can set a target GKI value to help track therapeutic status. Daily GKI values can be plotted to allow visual tracking of progress against an initial index value over monthly periods (**Figure 21**). Entrance into the predicted zone of brain tumor management would be seen as the GKI value falls below the set target value (as illustrated in **Figure 22**). Additionally, the GKIC can track the number of days that an individual falls within the predicted target zone.

## RESULTS

The GKIC was used to estimate the GKI for humans and mice with brain tumors that were treated with either calorie restriction or ketogenic diets (**Table 8**). The results show a clear relationship between the GKI and efficacy of dietary therapy using either the KD or calorie restriction (**Figure 23**). Therapeutic efficacy of the KD or calorie restriction is greater with lower GKI values than with higher values.

## DISCUSSION

The evidence presented shows that the GKI can predict success for brain cancer management in humans and mice using metabolic therapies that lower blood glucose and elevate blood ketone levels. Besides ketogenic diets, other dietary

therapies, such as calorie restriction, low carbohydrate diets, and therapeutic fasting, can also lower blood glucose and elevate bOHB levels and can have anti-tumor effects (Fine et al., 2012; Klement & Kammerer, 2011; Longo & Fontana, 2010; Mantis et al., 2003; Rieger et al., 2014; Safdie et al., 2009; Seyfried et al., 2003). The GKIC was developed to more reliably and simply predict therapeutic management for brain cancer patients under these dietary states than could measurements of either blood glucose or ketones alone. The data presented in **Table 8** support this prediction. As brain tumor cells are dependent on glucose for survival and cannot effectively use ketone bodies as an alternative fuel, a zone of metabolic management can be achieved under conditions of low glucose and elevated ketones. Ketone bodies also prevent neurological issues associated with hypoglycemia, which allows blood glucose levels to be lowered even further (Veech et al., 2001). Hence, ketone body metabolism can protect normal brain cells under conditions that target tumor cells. The zone of metabolic management is considered the therapeutic state that places maximal metabolic stress on tumor cells while protecting normal cells.

The GKI is useful in determining the success of dietary therapy to shift glucose- and lactate-based metabolism to ketone-based metabolism. As it is evidenced that shifting toward ketone-based metabolism underscores the utility of most dietary therapy in treating metabolic diseases and disorders (Meidenbauer & Roberts, 2014; Seyfried et al., 2008; Seyfried, 2014), the GKI can be used in determining the success of shifting metabolism in individual patients. The GKI

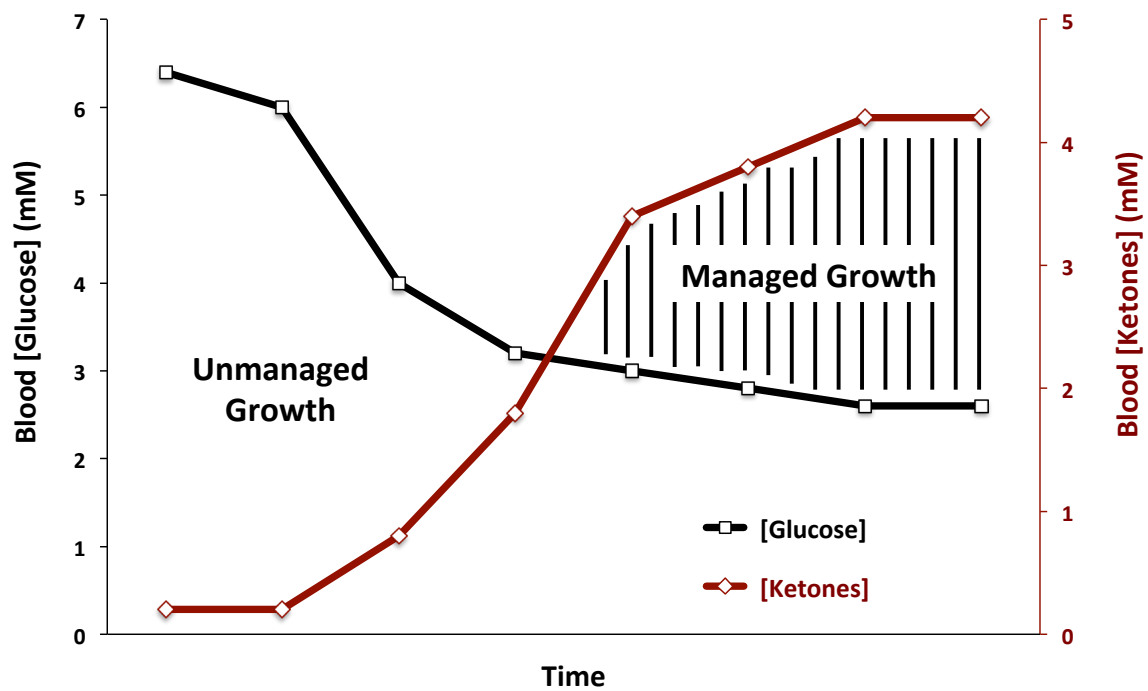
therefore can be used to study dietary therapy effectiveness in a large clinical population with a range of dietary strategies, with a composite primary endpoint consisting of lowering the subjects' GKI. This will allow investigators to parse the effects of successful dietary intervention on disease outcome from unsuccessful dietary intervention.

The zone of metabolic management is likely entered with GKI values between 1 and 2 for humans. Optimal management is predicted for GKI values approaching 1. To illustrate the utility that the GKI serves for an individual tracking their progress, **Figure 22** shows the glucose and ketone values from **Figure 19** plotted against their personal target metabolic zone of management. When an individual's GKI falls below the line denoting the target metabolic state, the zone of management is achieved.

The GKIC could have utility not only for management of brain cancer and possibly other cancers dependent on glucose and aerobic fermentation for survival, but also for other diseases or conditions where the ratio of glucose to ketone bodies could be therapeutic. Such disease and conditions may include Alzheimer's disease, Parkinson's disease, traumatic brain injury, and epilepsy (Seyfried, 2014). For example, the ketogenic diet has long been recognized as an effective therapeutic strategy for managing refractory seizures in children (Freeman & Kossoff, 2010). Therapeutic success in managing generalized idiopathic epilepsy in EL mice can also be seen when applying the GKI to the data presented on glucose and bOHB (Mantis

et al., 2004). Further studies will be needed to determine the utility of the GKIC for predicting therapeutic success in the metabolic management of disease.

**Figure 19.** Metabolic zone of tumor management in humans. As glucose falls and ketones rise, an individual enters the metabolic zone of management ('Managed Growth'). This can be tracked using the Glucose Ketone Index and the Glucose Ketone Index Calculator.





**Figure 20.** The Glucose Ketone Index Calculator unit converter. The GKIC converts glucose and ketone values from mg/dL to mM and from mM to mg/dL to increase compatibility across a range of glucose and ketone testing devices.

**Instructions:** Enter your blood glucose values (in mg/dL) and blood ketone ( $\beta$ -hydroxybutyrate) levels (in mmol) into the respective boxes to calculate your glucose/ketone index. Enter your desired glucose/ketone index value to help you keep track of your progress.

Glucose (mg/dL)	Ketones (mmol)	Glucose/Ketone Index
88	5.1	0.96
Desired Glucose/Ketone Index?		1

#### Glucose Units Converter

mg/dL	=	mmol
88	=	4.88
mmol	=	mg/dL
7.00	=	126.11

#### Ketone Units Converter

mg/dL	=	mmol
25	=	2.40
mmol	=	mg/dL
5.1	=	53.09

**Figure 21.** The Glucose Ketone Index Calculator tracking an individual's GKI with a target GKI of 1. The individual glucose and ketone values are displayed, along with the corresponding GKI values. The GKI values are plotted over the course of a month in black, whereas the target GKI value is plotted in red.

October				
Date	Glucose (mg/dL)	Ketones (mmol)	Glucose/Ketone Index	Desired Level?
1	61	7.4	0.46	Yes!
2	71	6.4	0.62	Yes!
3	80	5.9	0.75	Yes!
4	106	3.4	1.73	No
5	100	5.9	0.94	Yes!
6	83	6.2	0.74	Yes!
7	73	6.6	0.61	Yes!
8	69	6.6	0.58	Yes!
9	89	5	0.99	Yes!
10	76	6.6	0.64	Yes!
11	103	3.2	1.79	No
12	87	5.8	0.83	Yes!
13	70	4.9	0.79	Yes!
14	86	5.4	0.88	Yes!
15	68	6.3	0.60	Yes!
16	108	2.6	2.31	No
17	100	3.6	1.54	No
18	97	5.1	1.06	No
19	95	3.7	1.43	No
20	84	4.2	1.11	No
21	74	6.4	0.64	Yes!
22	78	6.6	0.66	Yes!
23	56	7.4	0.42	Yes!
24	96	6.3	0.85	Yes!
25	87	4.7	1.03	No
26	78	6.3	0.69	Yes!
27	112	7.4	0.84	Yes!
28	90	4.8	1.04	No
29	76	6.6	0.64	Yes!
30	84	5.9	0.79	Yes!
31	88	5.1	0.96	Yes!

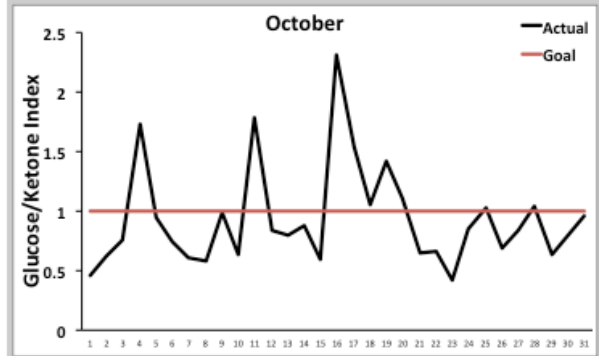
**Average**  
 Glucose 84.68  
 Ketone 5.56  
 GK Index 0.93

**Low**  
 Glucose 56  
 Ketone 2.6  
 GK Index 0.42

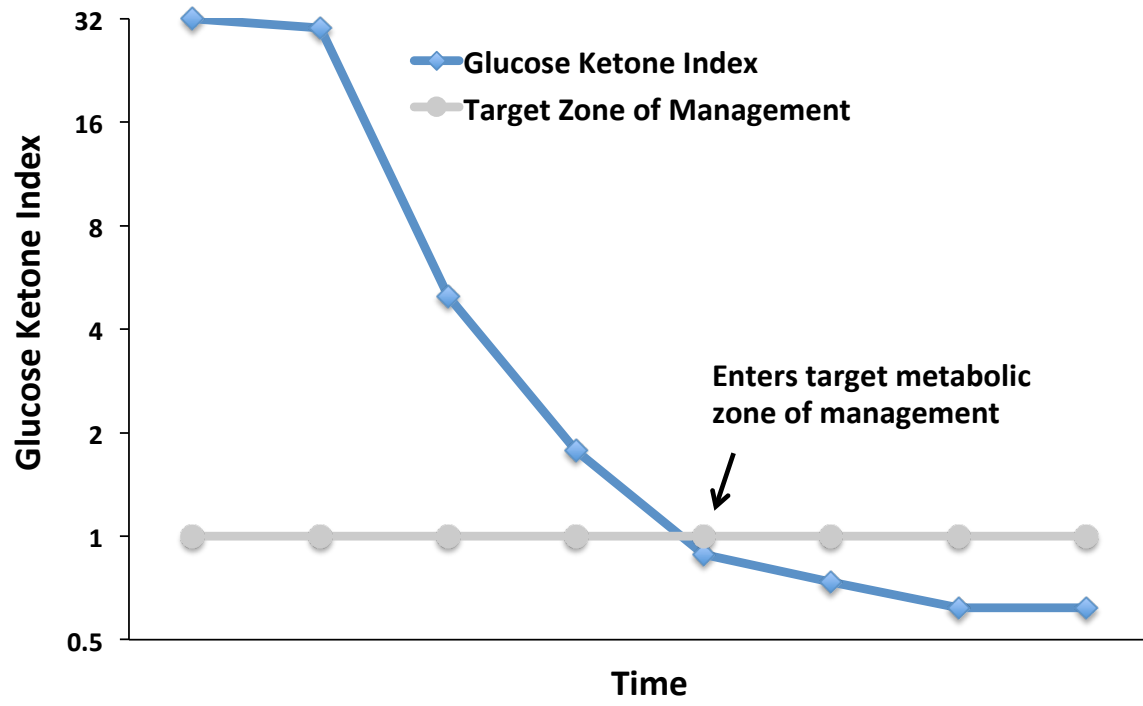
**High**  
 Glucose 112  
 Ketone 7.4  
 GK Index 2.31

**Range**  
 Glucose 56.00  
 Ketone 4.80  
 GK Index 1.89

**Time in Metabolic  
Zone of  
Management**  
 Yes 71.0%  
 No 29.0%



**Figure 22.** The Glucose Ketone Index Calculator tracks an individual entering their target metabolic zone of management. After an individual's Glucose Ketone Index falls below their target value, they have entered the metabolic zone of management. Tumor growth is slowed in the metabolic zone of management.



**Figure 23.** Effect of Glucose Ketone Index values on mouse brain tumor growth. Mice were implanted intracerebrally with syngeneic mouse CT-2A astrocytoma, and were put on an *ad libitum* or a calorie-restricted diet (40% daily calorie restriction) beginning 3 days after tumor implantation. Mice with lower GKI values had less brain tumor growth than mice with high GKI values. Modified with permission from Seyfried, Kiebish, Marsh, Shelton, Huysentruyt, & Mukherjee. (2011). *Biochimica et Biophysica Acta – Bioenergetics*. 1807(6), 577-594.



15.2

3.7

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Glucose Ketone Index



**Table 8. Low Glucose Ketone Index values are correlated with improved prognoses in humans and mice with brain tumors.**

Subjects	Tumor Type	Diet	# of Subjects	Days on Diet	Glucose (mM)	Ketones <sup>f</sup> (mM)	Glucose Ketone Index	Prognosis
Human <sup>1</sup>	Anaplastic Astrocytoma (Stage IV)	KD-UR <sup>a</sup>	1	0	5.5	0.2	27.5	No response to standard chemotherapy
				56	5.0	4.6	1.1	FDG uptake at tumor site was decreased by 21.77%; tumor margins were unchanged
	Cerebellar Astrocytoma (Grade III)	KD-UR	1	0	5.5	0.2	27.5	Tumor resected and initiated on KD while under standard chemotherapy, after tumor was radiologically stable by CT
				56	4.0	5.5	0.7	FDG uptake at tumor site was decreased by 21.84% <b>Notes:</b> Both patients remained in remission after return to standard diet for 5 years (Subject 1) and 4 years (Subject 2), at time of publication
Human <sup>2</sup>	Glioblastoma Multiforme (Grade IV)	KD-R <sup>b</sup>	1	0	7.5	0.2 <sup>g</sup>	37.5	Incomplete surgical resection of tumor; received chemotherapy and radiation therapy concurrent with diet
				21	3.5	2.5 <sup>g</sup>	1.4	No evidence of tumor by MRI after concurrent therapy <b>Notes:</b> Patient stayed on low calorie diet for an additional 5 months; tumor recurrence 3 months after low-calorie diet suspension
Mouse <sup>3</sup>	mouse CT-2A astrocytoma syngenic (C57BL/6J)	SD-UR <sup>c</sup>	7	13	9.1	0.6	15.2	Tumor dry weight: 55 ± 15 mg <sup>h</sup>
		SD-R <sup>d</sup>	6	13	5.2	1.4	3.7	Tumor dry weight: 7 ± 7 mg
		KD-UR	14	13	11.4	1.0	11.4	Tumor dry weight: 70 ± 15 mg
		KD-R	6	13	5.7	1.3	4.4	Tumor dry weight: 14 ± 8 mg
Mouse <sup>4</sup>	mouse CT-2A astrocytoma syngenic (C57BL/6J)	SD-UR	12-14	8	14.0	0.2	70.0	Tumor dry weight: 95 ± 25 mg <sup>h</sup>
		KD-UR	12-14	8	13.5	0.6	22.5	Tumor dry weight: 90 ± 15 mg
		KD-R	12-14	8	8.0	1.8	4.4	Tumor dry weight: 35 ± 5 mg
	human U87 glioma xenograft (SCID)	SD-UR	12-14	8	11.5	0.5	23.0	Tumor dry weight: 60 ± 10 mg <sup>h</sup>
		KD-UR	12-14	8	11.5	1.2	9.6	Tumor dry weight: 60 ± 7 mg
		KD-R	12-14	8	5.5	3.0	1.8	Tumor dry weight: 37 ± 5 mg
Mouse <sup>5</sup>	mouse GL261 astrocytoma (C57BL/6-cBrd/cBrd/Cr)	SD-UR	19	13	10.0	0.2	50.0	Median survival time: 23 days
		KD-UR	19	13	8.9	1.4	6.4	Median survival time: 28 days
		SD-UR + Rad <sup>e</sup>	11	13	9.7	0.3	32.3	Median survival time: 41 days
		KD-UR + Rad	11	13	9.7	1.7	5.7	Median survival time: 200+ days

<sup>1</sup>Nebeling LC, Miraldi F, Shurin SB, Lerner E. (1995) Effects of a ketogenic diet on tumor metabolism and nutritional status in pediatric oncology patients: two case reports. *J Am Coll Nutr*; 14:202-208.

<sup>2</sup>Zuccoli G, Marcello N, Pisanello A, Servadei F, Vaccaro S, Mukherjee P, Seyfried TN. (2010) Metabolic management of glioblastoma multiforme using standard therapy together with a restricted ketogenic diet: Case Report. *Nutr Metab (Lond)*; 7:33.

<sup>3</sup>Seyfried TN, Sanderson TM, El-Abbadi MM, McGowan R, Mukherjee P. (2003) Role of glucose and ketone bodies in the metabolic control of experimental brain cancer. *Br J Cancer*; 89:1375-1382.

<sup>4</sup>Zhou W, Mukherjee P, Kiebish MA, Markis WT, Mantis JG, Seyfried TN. (2007) The calorically restricted ketogenic diet, an effective alternative therapy for malignant brain cancer. *Nutr Metab (Lond)*; 4:5.

<sup>5</sup>Abdelwahab MG, Fenton KE, Preul MC, Rho JM, Lynch A, Stafford P, Scheck AC. (2012) The ketogenic diet is an effective adjuvant to radiation therapy for the treatment of malignant glioma. *PLoS One*; 7:e36197.

<sup>a</sup>Ketogenic Diet, Unrestricted

<sup>b</sup>Ketogenic Diet, Restricted

<sup>c</sup>Standard Diet, Unrestricted

<sup>d</sup>Standard Diet, Restricted

<sup>e</sup>Diet with Radiation therapy

<sup>f</sup>Blood/plasma beta-hydroxybutyrate measurement

<sup>g</sup>Urinary ketones were measured

<sup>h</sup>Mean ± 95% Confidence Interval

## CHAPTER SIX

### CONCLUSION

Dietary therapies, such as calorie restriction and the ketogenic diet, can delay or reduce symptoms associated with a range of age- and disease-related pathologies in basic and clinical studies. Restriction of caloric intake can promote longevity in a variety of species and can diminish adverse symptoms in a variety of preclinical models of neurological and non-neurological disorders, including epilepsy, Rett Syndrome, and brain cancer. Standardized guidelines can facilitate study validity and reproducibility with respect to dietary intervention as a primary and complementary therapeutic modality. Accordingly, my dissertation research discusses diet implementation guidelines for preclinical studies that adhere to standardized experimental design and biomarker monitoring in mouse models in order to maximize therapeutic efficacy, diet regimen safety, and cross-study data interpretability.

My dissertation research additionally investigated the effects of three diets on circulating plasma metabolites (glucose and  $\beta$ -hydroxybutyrate), hormones (insulin and adiponectin), and lipids over a 32-day period in C57BL/6J mice (Meidenbauer et al., 2014). The diets evaluated included a standard rodent diet (SD), a ketogenic diet (KD), and a standard rodent diet supplemented with fish-oil (FO). Each diet was administered in either unrestricted (UR) or restricted (R)

amounts to reduce body weight by 20%. The KD-UR increased body weight and glucose levels and promoted a hyperlipidemic profile, whereas the FO-UR decreased body weight and glucose levels and promoted a normolipidemic profile, compared to the SD-UR. When administered in restricted amounts, all three diets produced a similar plasma metabolite profile, which included decreased glucose levels and a normolipidemic profile. Linear regression analysis showed that circulating glucose most strongly predicted body weight and triglyceride levels, whereas calorie intake moderately predicted glucose levels and strongly predicted ketone body levels. These results suggest that biomarkers of health can be improved when dietary therapy is given in restricted amounts, regardless of macronutrient composition.

With the understanding of how to implement dietary therapy and how various dietary therapies affects glycolytic flux and biomarkers of health, my dissertation goes on to demonstrate an example of how to understand the mechanisms of how dietary therapy can manage disease (Meidenbauer & Roberts, 2014). Specifically, dietary therapy has been used to treat many individuals with epilepsy that are refractory to anti-epileptic drugs. The mechanisms for how dietary therapy confers seizure protection are currently not well understood (Danial et al., 2013). I evaluated the acute effects of glucose and  $\beta$ -hydroxybutyrate in conferring seizure protection to the EL mouse, a model of multifactorial idiopathic generalized epilepsy. EL mice were fed either a standard diet unrestricted or a calorie-restricted standard diet to achieve a body weight reduction of 20-23%. D-glucose, 2-deoxy-D-glucose, and  $\beta$ -hydroxybutyrate were supplemented in the drinking water of

calorie-restricted mice for 2.5 hours prior to seizure testing to simulate the effect of increased glucose availability, decreased glucose utilization, and increased ketone availability, respectively. Seizure susceptibility, body weight, plasma glucose, and  $\beta$ -hydroxybutyrate were measured over a nine-week treatment period. Additionally, excitatory and inhibitory amino acids were measured in the brains of mice using  $^1\text{H}$  NMR. Glutamate decarboxylase activity was also measured to evaluate the connection between dietary therapy and brain metabolism. I found that lowering of glucose utilization is necessary to confer seizure protection with long-term (>4 weeks) calorie restriction, whereas increased ketone availability did not affect seizure susceptibility. In the absence of long-term calorie restriction, however, reduced glucose utilization and increased ketone availability did not affect seizure susceptibility. Brain excitatory and inhibitory amino acid content did not change with treatment, and glutamate decarboxylase activity was not associated with seizure susceptibility. I demonstrated that reduced glucose utilization is necessary to confer seizure protection under long-term calorie restriction in EL mice, while acute ketone supplementation did not confer seizure protection. Further studies are needed to uncover the mechanisms by which glucose utilization influences seizure susceptibility.

My dissertation research also led to the development of a tool, the Glucose Ketone Index and related Glucose Ketone Index Calculator, to help track the efficacy of dietary therapy in clinical and preclinical populations. The Glucose Ketone Index Calculator is validated through the correlation between the Glucose Ketone Index

and prognosis of mice and humans with brain cancer. The Glucose Ketone Index Calculator can be commercialized to reach a broader population of individuals to help manage brain cancer.

## APPENDIX

### Publications Appearing in Dissertation

**Meidenbauer JJ** and Roberts MF (2014) Reduced glucose utilization underlies seizure protection with dietary therapy in epileptic EL mice. *Epilepsy & Behavior*. 39:48-54.

**Meidenbauer JJ**, Ta N, and Seyfried TN (2014) Influence of a ketogenic diet, fish-oil, and calorie restriction on plasma metabolites and lipids in C57BL/6J mice. *Nutrition & Metabolism*, 11:23.

## Other Publications

Mantis JG, **Meidenbauer JJ**, Zimick NC, Centeno NA, and Seyfried TN (2014) Glucose reduces the anticonvulsant effects of the ketogenic diet in EL mice. *Epilepsy Research*. 108(7):1137-1144.

Park D, **Meidenbauer J**, Sparta B, Seng WL, and McGrath P (2012) Zebrafish: A Predictive Model for Assessing Seizure Liability. McGrath (ed). *Zebrafish: Methods for Assessing Drug Safety and Toxicity*. John Wiley & Sons, Inc. Hoboken, NJ. 13:165-176.

Urits I, Mukherjee P, **Meidenbauer J**, and Seyfried TN (2012) Dietary Restriction Promotes Vessel Maturation in a Mouse Astrocytoma. *Journal of Oncology*, 2012:260439:1-10.

**Meidenbauer JJ**, Mantis JG, and Seyfried TN (2011) The EL Mouse: A Natural Model of Autism and Epilepsy. *Epilepsia*, 52(2):347-357.

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