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# The Effects of Even- and Odd-Numbered Medium Chain Triglyceride Ketogenic Diets on Autistic Behaviors in a Mouse Model

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TRINITY COLLEGE

**THE EFFECTS OF EVEN- AND ODD-NUMBERED MEDIUM CHAIN  
TRIGLYCERIDE KETOGENIC DIETS ON AUTISTIC BEHAVIORS IN  
A MOUSE MODEL**

BY

Lisa Saa

THESIS SUBMITTED TO  
THE FACULTY OF THE NEUROSCIENCE PROGRAM  
IN CANDIDACY FOR THE BACCALAUREATE DEGREE  
WITH HONORS IN NEUROSCIENCE

NEUROSCIENCE PROGRAM

HARTFORD, CONNECTICUT

May 8<sup>th</sup>, 2014

The Effects of Even- and Odd-Numbered Medium Chain Triglyceride Ketogenic  
Diets on Autistic Behaviors in a Mouse Model

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## **ACKNOWLEDGEMENTS**

I would like to thank Dr. Susan Masino for providing me with the opportunity to work on my thesis in her lab and guiding me throughout the entire process. I would also like to thank Dr. David Ruskin for his assistance with experimental design, data analysis, and moral support. Their encouragement and mentorship has been absolutely amazing and indispensable. Lastly, I would like to thank Jenny Nord and all the members of the Masino lab for their assistance during the study.

## **ABSTRACT**

**BACKGROUND:** A ketogenic diet (KD), which has restricted carbohydrates, sufficient protein, and a very high fat content, causes the body to switch from a glucose-based metabolism to a ketone-based metabolism. The KD has been effective at reducing seizures in epileptic patients. Autism is comorbid with epilepsy and characterized by restricted and repetitive behaviors, low sociability, and deficits in communication. A strict version of the long-chain triglyceride (LCT) KD has been effective at reducing autistic symptoms in BTBR T+tf/J, an autistic model of mice. However, a more moderate and clinically relevant version of the LCT KD has been shown to be ineffective in this model. Recent studies suggest that a KD derived primarily from medium chain triglycerides (MCTs) will effectively reduce the severity of autistic symptoms. A MCT KD has been shown to cause an increase in ketone bodies, acetyl-CoA, and ATP similar to a LCT KD. However, MCTs are hydrolyzed faster than LCTs and provide more kilocalories per gram of fat, allowing it to maintain clinical relevancy. Furthermore, only odd-numbered MCTs are anaplerotic substances, meaning that the metabolites of the Krebs cycle are refilled. Thus, the goal of the study was to determine a) if either even- or odd-numbered MCTs result in a beneficial alleviation of autistic symptoms in mice and b) the role of anaplerosis in modifying behavior and establishing ketosis.

**METHODS:** BTBR T+tf/J mice were given one of the following metabolic treatments for three weeks: pellet chow (control), pellet chow balanced with 17% cellulose, 17% heptanoic acid KD (odd-numbered MCTs), and 17% octanoic acid (even-numbered MCTs). A battery of behavioral tests was given in order to quantify the core symptoms of autism, including deficits in sociability, communication, and motor control as well as self-directed repetitive behaviors.

Furthermore, glucose and ketone blood analysis were conducted in order to elucidate the mechanisms of the diet.

**RESULTS:** The mice in the pellet and cellulose control groups did not clearly exhibit autistic behaviors as expected, primarily in the three-chamber test for sociability and self-directed repetitive behavior. However, the heptanoic and octanoic treatment groups spent significantly more time engaged in frontal contact than the cellulose control.

**CONCLUSION:** The results of the current study are conflicting and inconclusive. The effects of even- and odd-numbered MCT KDs should be investigated and expanded further in order to understand the impact of this metabolic treatment on alleviating autistic symptoms.

## INTRODUCTION

### *Autism*

Autism Spectrum Disorders (autism) are pervasive developmental disorders characterized by deficits in social communication and fixated interests or repetitive behavior. The Diagnostic and Statistical Manual (DSM) is published by the American Psychiatric Association and lists the standard criteria used to classify and diagnose mental health disorders. The DSM-IV differentiated between autistic disorder, Asperger's disorder, childhood disintegrative disorder, and pervasive developmental disorder not otherwise specified. However, the recently published DSM V has now included all of those diagnoses under the criteria for autism (APA, 2013). It is estimated that autism is prevalent in 70-90/10,000 people or about 1 child out of 125 (French et al., 2012). Although the prevalence of the disorder has increased significantly from the first reported value of 4.1/10,000 or about 1 child out of 2,440 (Lotter, 1966), the increase of prevalence may not be directly linked to an increase in incidence. Evidence suggests that changes in diagnostic criteria and increased awareness of symptoms of autism are at least partly responsible for the surge (French et al., 2012). Autism is about five times more prevalent in boys than girls (CDC, 2014). However, some have argued that this ratio might be overstated; rather, girls with autism may display different behavioral characteristics and as such, are vastly underdiagnosed (Wing, Gould, & Gillberg, 2011; Kopp & Gillberg, 1992).

### *Symptoms of Autism*

Autism is extremely heterogeneous in the biological, cognitive, and behavioral domains. This has been illustrated in studies focused on identical twins with autism who do not have identical symptoms (Kim & Lord, 2012). There are, however, broad generalities that span the



diagnosis of autism. Behaviorally, autism is typically characterized by deficits in communication, socialization, sensory reactivity, and motor skills (Barton et al., 2012). Cognitively, about 20-50% of children with autism have an intellectual disability or are classified as mentally retarded (Charman et al., 2011; Chakrabarti & Fombonne, 2005). Furthermore, about 70% of people with autistic disorder have an IQ of below 70 (Coleman, 2000).

Autism is comorbid with a multitude of other behavioral and structural disorders. One study, which examined comorbidity rates with psychiatric disorders, had a sample size of 112 ten- to fourteen-year old children diagnosed with autism (Simonoff et al., 2008). They found that 70% had at least one comorbid disorder and 41% had two or more, with the most common ones being social anxiety disorder (29.2%), ADHD (28.2%), and oppositional defiant disorder (28.1%). Another study found comorbidity rates of 44%, 37%, and 32% for specific phobias, obsessive compulsive disorder, and ADHD, respectively (Leyfer et al., 2006). Furthermore, autism is comorbid with epilepsy in 7% to 42%, with more studies suggesting that about one-third of those with autism are also epileptic (Danielsson et al., 2005). Children with autism may also experience gastrointestinal symptoms, food sensitivities and allergies, and autoimmune abnormalities (Weber & Newmark, 2007) as well as marked reductions in omega-3 fatty acids and polyunsaturated fatty acids (Vancassel et al., 2001). They have also been found to have a smaller corpus callosum (Egaas, Courchesne, & Saitoh, 1995), and lower relative glucose metabolic rates bilaterally in the thalamus and ventral caudate (Haznedar et al., 2006). fMRI studies have shown abnormalities in the fusiform face area (Stigler & McDougle, 2012). Biological markers and early brain abnormalities in children with autism have been reported, but they are not yet reliable enough to be used in diagnoses (Barton et al., 2012).

Because of these factors, autism is a behavioral diagnosis that can be made at significantly different time points. For example, children with autism may start to show disturbances of movement indicative of autism as early as 4-6 months, or even at birth (Teitelbaum et al., 1998). However, some toddlers may have regressive autism, meaning they experience normal development and do not show autistic symptoms, as measured by developmental delays in social communication, behavior, and sensory abilities, by their first birthday. However, they begin to display autistic symptoms between the ages of one and two, suggesting that a second screening is necessary by the second birthday (Landa, Holman & Garrett-Mayer, 2007).

### *Causes of Autism*

Autism is believed to be caused by genetic and non-heritable factors. About 20% of people with autism have a genetic etiology, and about 5% show chromosomal aberrations (Betancur & Coleman, 2012). Furthermore, the prevalence of autism is greater in monozygotic twins than in dizygotic twin pairs, with 90% and 30% concordance rates, respectively (Folstein & Rutter, 1977; Bailey et al., 1995). Furthermore, the prevalence of autism in siblings is 3%, and the rate decreases as the degrees of separation between family members increases (Smalley, Asarnow & Spence, 1988). In a thorough review, 103 disease genes and 44 recurrent genomic imbalances were reportedly found in patients with autism (Betancur & Coleman, 2012). Taken together, the etiology of autism is at least partially based in genetics. However, it may not be a single gene that is responsible for autism; instead, each gene may be a risk factor for a component of the disease, with the risk of autism increasing as the number of genes involved increases (Dawson et al., 2002).

In a meta-analysis of epidemiological studies, non-heritable factors were found to be associated with autism, although it is strongly believed that autism is predominately genetically determined (Sandin et al., 2012). In terms of parental age, the risk of autism in offspring of mothers who are 35 years or older is 1.3, compared to mothers who are 25-29 years old. The risk of autism in offspring of fathers who are 40 years or older is 1.4, compared to fathers aged 20-29 years, the risk is 1.7 for fathers who are 50 years or older. The risk also increases with low-birth weights, pre-term births, and fetal growth restriction. Furthermore, the etiology of autism may be affected by prenatal exposure to infectious agents, certain maternal medications, prenatal nutrition status, maternal lifestyle, and exposure to environmental chemicals (Lyll, Schmidt & Hertz-Picciotto, 2012). Considering the perinatal and prenatal associations and genetic variants, autism may be strongly linked to epigenetic factors (Persico & Bourgeron, 2006.)

Autism may also be related to metabolic deficiencies and mitochondrial defects. In a comprehensive literature review examining mitochondrial dysfunction, children with autism had a 5.0% prevalence rate while the general population has a rate of approximately 0.01%, making it the most common metabolic abnormality associated with autism (Rossignol & Frye, 2012; Frye & Rossignol, 2011). However, the prevalence rate of mitochondrial disorders could be underestimated due to differences in clinical techniques (Giulivi et al., 2010). In a study comparing 80 autistic and 73 control children, the autistic children had decreased antioxidant abilities and increased oxidative stress (James et al., 2006). Another study found that autistic children aged 5-16 years had vitamin insufficiencies and reduced capacities for energy transport, sulfation, and detoxification (Adams et al., 2011). Furthermore, children with autism showed lower serum carnitine and pyruvate levels and increased ammonia and alanine levels, further suggesting a mitochondrial defect (Oliveira et al., 2005).

## *Treatment Options for Autism*

Currently, there is no treatment for autism. However, there are treatments for certain symptomatic aspects. Behavioral therapy is the most widely used treatment option for the symptoms of autism (Soorya, Carpenter & Warren, 2012). Behavioral therapy uses reinforcement theory to teach new skills and reduce problematic behaviors (Lovaas, 1987). There are specific interventions that address social impairments, communication deficits, and psychiatric problems (Soorya et al., 2012). For instance, speech therapy is recommended for those with language deficits, and sensory and auditory integration therapy is recommended for sensory deficits (Weber & Newmark, 2007). Furthermore, behavioral therapy through virtual reality has been used as a learning aid to help those with autism interact with the world (Strickland, 1997).

For those with autism who are also known to have a metabolic abnormality, targeting the metabolic defect via dietary and vitamin treatments has been shown to be effective (Page, 2000). For instance, those who have hyperuricosuric autism, or infantile autism combined with a 24-hour urate excretion greater than two standard deviations above the mean, have benefited from a low purine diet (Page, 2000). Autistic children with urinary peptide abnormalities were treated with a gluten-free, casein-free (GFCF) diet for a year and experienced reduced autistic behavior, specifically increased social interactions, improved language skills, and increased interest in the environment (Knivsberg et al., 2003). However, in a randomized, double blind study of 15 autistic patients, the GFCF diet had no effect on alleviating autistic symptoms (Elder et al., 2006). Furthermore, omega-3 fatty acid supplementation showed significant increases in language skills and aggressive behavior in autistic children who were not tested for metabolic defects (Weber & Newmark, 2007) and combined pyridoxine and magnesium supplementation

improved (but did not completely reverse) autistic symptoms (Page, 2000), suggesting an overall beneficial role of nutrient supplementation and dietary treatment in autistic children. However, many dietary and nutrient studies have not been rigorously tested, indicating the need for more research in this field.

### ***Ketogenic Diet***

The ketogenic diet (KD) is comprised of restricted carbohydrates, sufficient protein, and a very high fat content. It mimics the effects of fasting by minimizing glucose metabolism and producing and promoting blood ketones. Ketones become the primary source of metabolic fuel for the brain, bypassing glycolysis, and producing ATP at a much higher rate, causing the body to enter a state of ketosis (Ruskin et al., 2009).

The KD was developed in 1921 by Wilder as a treatment for intractable epilepsy. Although its use has waned after the introduction of anti-epileptic drugs (AEDs), a multitude of studies have demonstrated the efficacy of the diet as a treatment for epilepsy when AEDs do and do not work. In a multicenter study, it was found that at least 50% of epileptic patients given a KD experienced a  $\geq 50\%$  reduction in seizures (Vining et al., 1998). In a meta-analysis establishing the efficacy of the KD, 24% of those who adhered on the diet past three months achieved complete seizure control and 52% of adherers achieved  $\geq 90\%$  seizure control (Henderson et al., 2006). In a three- to six-year follow up on children who were on the diet, 13% were seizure-free, 14% had a  $\geq 90\%$  decrease in seizures, and 19% were free of medications (Hemingway et al., 2001). In the first randomized control trial, epileptic children who were placed on the diet had significantly lower baseline seizures and greater seizure reduction compared to the control group (Neal et al., 2008).

The diet is slowly administered over the course of five days, according to the Johns Hopkins protocol. Before the start of the diet, the patient is directed to minimize carbohydrate intake and begin fasting after dinner. The patient is admitted on the first day, and blood glucose is measured to ensure levels do not drop below 25 mg/dL. At dinner, the patient is given one-third of the expected full meal. Over the next four days, the quantity of the KD is advanced in one-third increments (Hartman & Vining, 2007). Medications are tapered off as the effects of the KD begin to take place, usually within 3-6 months of the diet's introduction. Because the diet requires strict compliance for ketosis to be maintained, the KD is a difficult treatment to implement and often used as a last resort, despite its proven efficacy.

#### *Mechanisms of the KD*

The mechanism by which the KD exerts anticonvulsant and antiepileptic effects is not completely understood, although there have been many proposed theories. In one experiment, adolescent rats were placed on a control or KD for three weeks (Bough et al., 2006). Microarray technologies were then used to understand gene expression in the hippocampi, and the data illustrated that there was upregulation of the genes that encode for energy metabolism enzymes, suggesting that the KD works by altering mitochondrial gene expression to improve energy reserves. Moreover, a multi-analysis examining the anticonvulsant efficacy of the KD indicated that the metabolic adaptations to the KD are fundamentally responsible (Bough & Rho, 2007). Others hypothesize that raised levels of plasma polyunsaturates (Cunnane et al., 2002), elevated GABA levels (Yudkoff et al., 2008), diminished reactive oxygen species production (Sullivan et al., 2004), or reductions in glutamate and increased ketone bodies (Freeman et al., 2007) may be responsible for the neuroprotective effects of the KD.

Our lab has explored the involvement of increased adenosine levels (Masino et al., 2011a; Masino et al., 2011b). An in vivo study of mice on the KD illustrated that increased intracellular ATP concentrations resulted in reduced extracellular glucose in the hippocampal CA3 region. The neuronal ATP was released via pannexin channels, and the ATP was dephosphorylated into adenosine. The resultant activation of the adenosine A1 receptors led to the opening of the K<sup>+</sup> channels (Kawamura et al., 2010). In another study, parents were given a questionnaire to assess child behavior after participation in events that would increase, decrease, or have no influence on adenosine levels. Beneficial effects were observed when children participated in behaviors that increased adenosine levels (Masino et al., 2011a). Discovering the precise mechanism by which the KD is effective would elucidate etiologies of neurological disorders that benefit from the KD.

#### *Autism and the KD*

As mentioned prior, autism is often comorbid with epilepsy (Buitelaar & Willemsen-Swinkels, 2000). This, along with the suggested neuroprotective and neuromodifying effects of the KD, initiated interest in testing the efficacy of alleviating autistic symptoms with a KD. A pilot study by Evangelidou et al. (2003) applied the KD to thirty children between the ages of four and 10. The diet was administered for six months intermittently. All of those who adhered to the diet reported significant (11%), average (44%), or minor (44%) improvements on the Childhood Autism Rating Scale. On online survey examining the efficacy of AED and non-AED treatments, only the KD, Atkin's or modified Atkin's diet, gluten-free casein-free diet, and hyperbaric oxygen therapy were perceived to improve both seizures and behavior (Frye, Sreenivasula, & Adams, 2011).

In animal studies, when a strict KD (6.6:1 ratio of fats to proteins and carbohydrates) was given to a mouse model of autism, impaired sociability, repetitive behaviors, and communication deficits were alleviated, suggesting that the KD may be used as a therapy for autism (Ruskin et al., 2013). However, when a moderate KD (3.2:1 ratio of fats to proteins and carbohydrates) was given to a mouse model of autism, the beneficial alleviation of autistic symptoms was not observed (Reuman, 2013). In the latter study, the glucose levels were not significantly decreased compared to the control group whereas they were in the former study. However, the levels of ketones were increased in both studies. This suggests that the resultant increase in ketone bodies and/or decrease in glucose levels may be responsible for the beneficial aspects of the KD. As such, a KD that maintains high ketone levels and low glucose levels, while maintaining clinical relevancy, could be potentially used as a treatment option for children with autism.

#### *Medium-Chain Triglyceride KD*

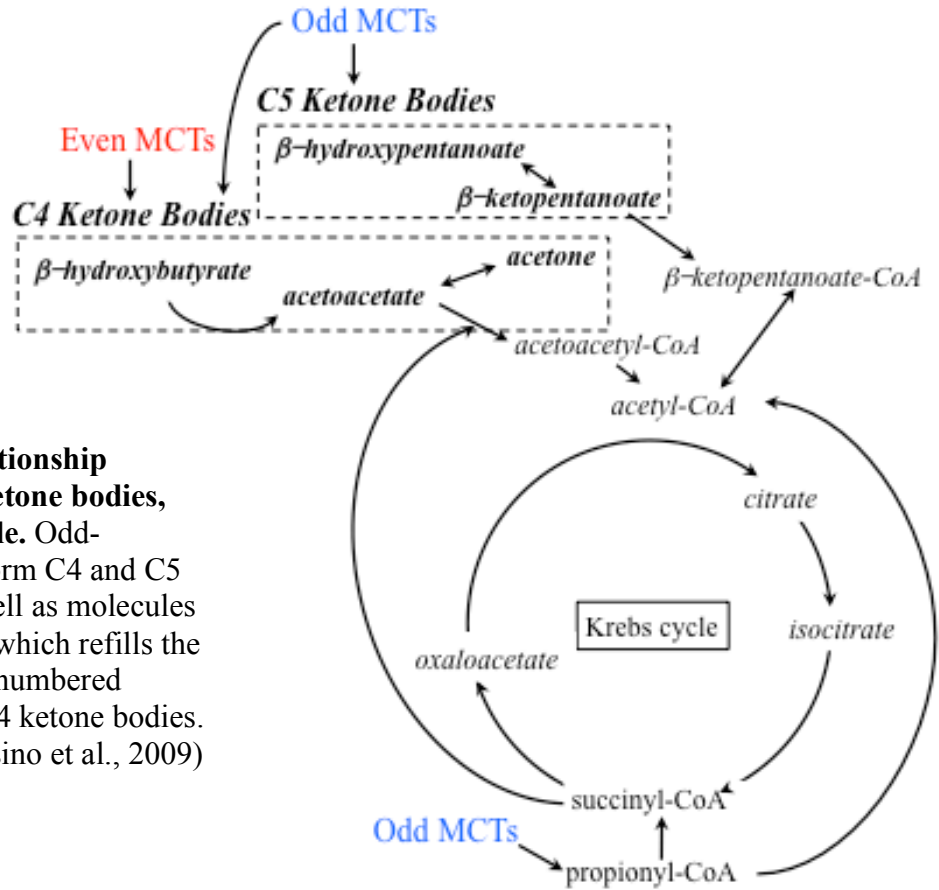
The classic KD primarily uses long-chain triglycerides (LCTs) as the source of fat and has a ratio of 3:1 - 4:1 fats to combined proteins and carbohydrates, with the majority of calories coming from fats ( $\geq 90\%$ ). Because of the high consumption of fats required for the body to enter ketosis, the diet is not very palatable and can result in undesired side effects, such as constipation and vomiting. In contrast, a KD comprised of medium-chain triglycerides (MCTs) requires fewer calories from fat ( $\sim 35\%$ ) for the body to enter ketosis and may be adopted as a more clinically relevant alternative to the LCT KD.

MCTs are fatty acids with carbon chains ranging from approximately 7 to 12 carbons. They are usually in the form of oils or soft solids at room temperature. Natural sources of even-numbered MCTs include butter and coconuts. Odd-numbered MCTs are rarely found in nature but can be synthesized from natural oils.



## Mechanistic basis and clinical relevance of the MCT KD

MCTs can be used in place of, or in conjunction with, LCTs in order to establish ketosis while maintaining clinical relevance (Labarthe & Des Rosiers, 2008). MCTs have been shown to cause an increase in ketone bodies, acetyl-CoA, and ATP (de Baulny & Superti-Furga, 2006; Deng et al., 2009; Borges & Sonnewald, 2012; Brunengaber & Roe, 2006). However, only odd-numbered MCTs are anaplerotic substances. Odd-numbered MCTs are metabolized by  $\beta$ -oxidation to produce propionyl-CoA. Propionyl-CoA oxidation results in succinyl-CoA and oxaloacetate, which refill the Krebs cycle (de Baulny & Superti-Furga, 2006). Furthermore, propionyl-CoA can produce two molecules of acetyl-CoA, which also refills the Krebs cycle. The excess carbons are then metabolized by the liver into C4 (acetoacetate and R- $\beta$ -hydroxybutyrate) and C5 ( $\beta$ -ketopentanoate and  $\beta$ -hydroxypentanoate) ketone bodies (Borges & Sonnewald, 2012; Brunengaber & Roe, 2006; Labarthe & Des Rosiers, 2008). In contrast, even-numbered MCTs are metabolized only into acetyl-CoA molecules and C4 ketone bodies; they do not refill the Krebs cycle (de Baulny & Superti-Furga, 2006; Deng et al., 2009). Figure 1 provides a brief mechanistic schematic of the way even- and odd-numbered MCTS are metabolized.



**Figure 1: The relationship between MCTs, ketone bodies, and the Krebs cycle.** Odd-numbered MCTs form C4 and C5 ketone bodies as well as molecules of propionyl CoA, which refills the Krebs cycle. Even-numbered MCTs only form C4 ketone bodies. (Adapted from Masino et al., 2009)

MCTs are hydrolyzed faster and better absorbed than LCTs (Labarthe & Des Rosiers, 2008). Furthermore, MCTs produce more ketones per kilocalorie than LCTs allowing for a greater allowance of proteins and carbohydrates making it more clinically relevant (Kossof, Zupec-Kania, and Rho, 2009). As such, patients who are on the MCT KD have an improved tolerance to the diet as well as fewer negative side effects, such as constipation, compared to the LCT KD (Kossof et al., 2009). However, other studies have reported abdominal bloating and diarrhea associated with the MCT KD (Schwartz et al., 1989; Sills et al., 1986). Table 1 provides an example of a LCT KD and MCT KD meal for comparison purposes while Table 2 provides the macronutrient composition of a standard diet, strict LCT KD, moderate LCT KD, and MCT KD given to mice.

**Table 1: Example LCT KD and MCT KD meals** (Kossof, Zupec-Kania, & Rho, 2009; Liu & Wang, 2013)

LCT KD Dinner	MCT KD Lunch
35g 36% heavy cream	30g brown rice
<u>Ground beef and cheese:</u> 11g ground beef 10g cheese 8g butter	<u>Salad:</u> 31g lettuce 46g sliced cucumber 26g sliced tomatoes 35g alfalfa sprouts
26g cooked broccoli	30g grilled chicken cooked in 3g canola oil
11g butter	62.5g skim milk mixed with 18.1g MCT oil
<b>1100 kcal diet</b> <b>90% calories from fat</b>	<b>1500 kcal diet</b> <b>71% calories from fat</b>
<b>4:1 ratio of fats to protein and carbohydrates</b>	<b>2.4 :1 ratio of fats to protein and carbohydrates</b>

**Table 2: Macronutrient composition of various KD used in rodent studies.** Approximately 90% of calories come from fat in the moderate LCT KD whereas about 35% of calories come from fat in the MCT KD (Willis et al., 2010). As a result, the strict LCT KD has the greatest ratio of fats to protein and carbohydrates; it is 41.25 times greater than the standard diet. In contrast, the MCT KD has a ratio of fats to proteins and carbohydrates that is only 2.4 times greater than the standard diet.

	Standard Diet	Strict LCT KD	Moderate LCT KD	MCT KD
<b>Fats</b>	13.5%	76.7%	65.8%	28.2%
<b>Proteins</b>	28.5%	8.5%	18.1%	23.6%
<b>Carbohydrates</b>	58.0%	3.2%	3.6%	48.1%
<b>Ratio of fats to proteins and carbohydrates</b>	0.16:1	6.6:1	3.2:1	0.39:1

## Diseases of the CNS and the MCT KD

Past and recent studies have indicated that a MCT KD is potentially an effective treatment for various diseases of the central nervous system (CNS), including epilepsy, Huntington's disease, and Alzheimer's (Kim et al., 2012; Willis et al., 2010; Huttenlocher et al., 1971; Mochel et al., 2010; Nguen et al., 2013; Aso et al., 2013). In a genetic mouse model of generalized epilepsy, triheptanoin (at a 35% caloric intake) reduced seizure susceptibility and did not alter blood glucose (Kim et al., 2012). In a study using pilocarpine-induced status epilepticus CF1 mice (SE), triheptanoin (at 35% caloric intake for up to 7.5 weeks) was fed to SE and control mice; triheptanoin was anticonvulsant in the SE mice and the corneal kindling seizure mouse model (Willis et al., 2010). In children and adolescents with intractable epilepsy, a MCT diet (octanoic and decanoic acids) decreased rate of seizures (Huttenlocher et al., 1971). In another study, fifty children with drug resistant epilepsy were treated with a MCT emulsion diet (to increase palatability), eight achieved complete control of seizures, and fourteen had seizures reduced by 50-90% with the addition of anticonvulsants (Sills et al., 1986).

Triheptanoic oil (at 40% caloric intake) was given to six patients with Huntington's disease for five days, and an increase in serum IGF1 was observed. Because IGF1 is associated with inhibiting mutant huntingtin toxicity, the increase may restore huntingtin function and have beneficial effects on those with Huntington's disease (Mochel et al., 2010). Furthermore, Huntington's, in addition to other neurodegenerative diseases, affects motor control and movement. In one study, Sprague-Dawley rats were subjected to aortic banding, resulting in pressure-overload induced cardiac hypertrophy. After one week, they were treated with triheptanoin, which was shown to attenuate their hypertrophy and improve diastolic function and cardiac glucose oxidation (Nguen et al., 2013).

In Alzheimer's patients who lack the APO-ε4 gene (one of the risk factors for Alzheimer's), MCT treatment increased cognitive performance on the Alzheimer Disease Assessment Scale-Cognitive subscale, and for all subjects, higher ketone values were positively correlated with improvements in paragraph recall; hyperketonemia, as induced by MCTs, increased cognitive functioning in patients with Alzheimer's (Reger et al., 2003). Another study examined the effect of a LCT KD supplemented with MCTs on a familial Alzheimer's mouse model. The treatment resulted in improved cognition, illustrated anti-inflammatory properties of ketone bodies, and improved mitochondrial status (Aso et al., 2013).

Because of the effectiveness MCTs have displayed in diseases of the CNS, specifically epilepsy as it is comorbid with autism, it is reasonable to assume that a state of ketosis as induced by MCTs would result in beneficial effects in a mouse model of autism. Furthermore, the diet is much more clinically relevant as it allows for a greater allowance of proteins and carbohydrates, suggesting that it would be more palatable and result in higher levels of compliance.

### ***BTBR T+tf/J Mouse Model of Autism***

#### *Physical and Genetic Characteristics*

BTBR mice are an inbred strain of mice carrying the wildtype T (brachyury) gene and the tufted hair (tf) mutation. They have been shown to exhibit genetic and physical characteristics similar to those with autism. In regards to brain anatomy, BTBR mice have an absent corpus callosum, and a reduced corpus callosum was present in individuals with autism (Wahlsten, Metten, & Crabbe, 2003; Egaas, Courchesne, & Saitoh, 1995). Furthermore, BTBR mice have reduced BDNF mRNA levels, primarily in the dentate gyrus, as well as decreased neurogenesis in the hippocampus (Silverman et al., 2006; Stephenson et al., 2011). Similarly, those with

autism have hippocampal abnormalities, namely reductions in the right posterior hippocampus (Nicolson et al., 2006). The hippocampus is important for memory, emotional behavior, and information processing; deficits in all three of these areas are characteristic of patients with autism.

In terms of genetics, BTBR mice have one gene that expresses a single nucleotide polymorphism (SNP) which differentiates them from the B6 control mice group. The *Kmo* gene encodes for kynurenine 3-hydroxylase, an enzyme that regulates the metabolism of kynureic acid (Meyza et al., 2003; McFarlane et al., 2008). Kynureic acid, an antagonist of glutamate and nicotine receptors, may promote neuroprotection, dendritic spine formation, and dopamine release (McFarlane et al., 2008). Altered levels of kynureic acid were also found in patients with schizophrenia, Huntington's disease, and Alzheimer's disease (Linderholm et al., 2012; Beal et al., 1992; Guillemin & Brew, 2002). Furthermore, BTBR mice have a deletion of the *Disc1* mutation, which has been linked to male patients with Asperger syndrome and Schizophrenia (Kilpinen et al., 2007).

### *Behavioral characteristics*

Through a number of studies, BTBR mice have been shown to exhibit behavioral deficits that are similar to the core symptoms of autism. BTBR mice display deficits in social interactions, increased levels of self-directed repetitive behaviors, and impairments in communication and motor control.

In regards to sociability, BTBR mice have shown decreased sociability in the three-chamber sociability test and impaired juvenile play compared to control mice (McFarlane et al., 2008; Moy et al., 2004; Silverman et al., 2010). In studies that assess repetitive behaviors, BTBR mice are found to groom more than control mice (McFarlane et al., 2008; Pearson et al., 2011;

Silverman et al., 2010). However, it has been noted that this response may be normal as this strain of mice has a mutation in the tufted gene, resulting in hair loss; as such, the increased grooming behaviors might be a natural response to increased itching (Pearson et al., 2011). Additionally, BTBR mice have shown communication deficits comparable to those with autism. BTBR mice produce an unusual pattern of vocalization that is similar to atypical vocalizations produced by infants with autism (Scattoni et al., 2008). Male BTBR mice display minimal ultrasonic vocalization responses and reduced scent marking when exposed to female urine (Wöhr, Rouillet, & Crawley, 2011). Furthermore, the BTBR mice fail to socially transmit food preferences to their cage mates (McFarlane et al., 2008). Lastly, BTBR mice demonstrate low levels of performance on the rotarod task, which is a measure of motor coordination and balance; patients with autism may demonstrate impaired motor learning, including problems with coordination and balance and gait disturbances (Moy et al., 2004; Jansiewicz et al., 2006; Kielinen et al., 2004).

### ***Thesis Overview and Hypothesis***

The success with the strict LCT KD on alleviating autistic symptoms in the BTBR mouse model of autism is a very promising study that illustrates the potential for novel therapies to treat the symptoms of autism. However, the more moderate LCT KD was not able to replicate these results. Although the mechanism by which the KD works is unknown, recent evidence suggests that ketosis may lead to improved behavioral symptoms of autism. As such, it is reasonable to assume that a MCT KD would produce a similar level of ketosis and effectiveness at ameliorating autistic symptoms in a mouse model of autism, while maintaining clinical relevance. To analyze this theory, we studied the behavior of BTBR mice fed an odd-numbered

MCT KD, an even-numbered MCT KD, a diet balanced with cellulose, and a control diet pellet diet.

The current study was designed to (1) investigate the efficacy of a MCT KD on ameliorating autistic symptoms in a mouse model of autism, and (2) determine the effect of anaplerosis and the potential role it plays in the mechanistic action of the KD.

(1) We hypothesized that BTBR mice fed a 17% odd-numbered MCT KD (heptanoic acid) and a 17% even-numbered MCT KD (octanoic acid) would have improved sociability, motor, and communication skills and decreased repetitive behaviors. This was based on studies that suggest a MCT KD is more effective than a LCT KD in terms of metabolism as well as the assumption that the mice would undergo a similar level of ketosis as the mice treated with a strict LCT KD.

(2) Recent studies suggest that anaplerosis is more effective than ketosis alone in producing ATP, ketone bodies, and acetyl CoA. Therefore, we predicted that the odd-numbered MCT KD would be more effective than the even-numbered MCT KD at ameliorating autistic symptoms in the BTBR mouse model of autism.

## **METHODS**

### *Animals and test procedures*

BTBR (Jackson Laboratories) male mice were weaned at P21. Two to six animals were housed in cages with enrichment toys, given food and water ad libitum, and kept on a 12hr:12hr light:dark cycle. Starting at five weeks of age, the mice were randomly assigned to one of the following groups: 17% cellulose, 17% heptanoic acid, 17% octanoic acid, and pellet chow (LabDiet 5001). The first three diets were created by mixing powdered pellet chow and, by



weight, 17% heptanoic acid (oil), 17% octanoic acid (oil), and 17% cellulose powder, respectively. Water was added to the mixture, and pellets were then hand-formed and dehydrated on high heat for 12-18 hours. The mice were placed on the dietary treatment for three weeks before behavioral testing began. The heptanoic and octanoic treatment had a ratio of 0.39:1 fats to carbohydrates and proteins whereas the pellet and cellulose diets had a ratio of 0.16:1.

Weight, food intake, ketone levels, and glucose levels were recorded. Four different behavioral tests were run: the three-chamber sociability test measured social interaction skills of mice, the single-grooming test assessed self-directed repetitive behaviors, the social transmission of food preference (STFP) evaluated communication skills, and rotarod testing measured relative motor control. Behavioral testing was performed between eight and ten weeks of age between 10:00 am and 4:00 pm.

#### *Weight and food intake*

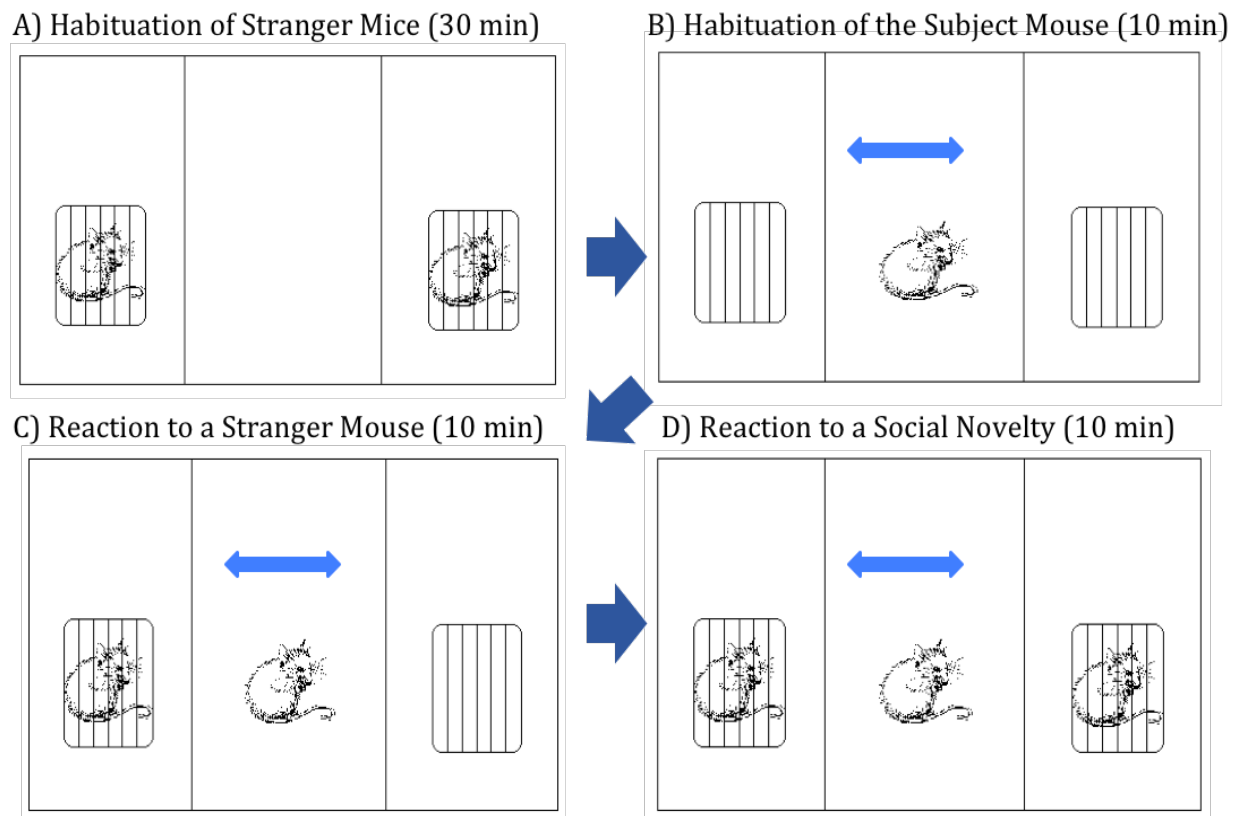
Body weights of each mouse were measured before treatment (five weeks of age) as well as before any testing began (eight weeks of age). Daily food intake of each cage was measured weekly, and then divided among the number of mice per cage to derive an estimate of how much each mouse ate. All four weeks of data was collapsed across time to approximate the amount of food eaten on a daily basis over the three weeks per mouse.

#### *Ketone and glucose levels*

Blood glucose and ketone ( $\beta$ -hydroxybutyrate) levels were measured before treatment began and at eight weeks of age. The mice were anesthetized by placing them in a small plastic container with isofluorane. Blood was then taken from the tail vein using a razor blade. The mice were placed back into their home cage to recover. The blood was analyzed using a Precision Xtra glucose/ketone meter and strips.

### *Three-chamber sociability test*

The three-chamber sociability test quantifies preference for social interactions and social novelty (Crawley, 2004; Moy et al., 2004). The apparatus was made of Plexiglas and consisted of three chambers of equal size (42.5 cm X 19.1 cm X 22.2 cm). Two side chamber doors with glass blockers were present. They were used to separate the center chamber from the side chambers. A wire cage (inverted pencil cup) was placed in the far back corners of the side chambers. A cement block sat atop the wire cages. The cages and blocks were present during the entirety of testing.



**Figure 2: The three-chamber sociability test.** **A)** Two stranger mice habituate to their individual cages for thirty minutes. **B)** The subject mouse habituates to the center chamber for ten minutes with the side chamber doors closed. The doors are then opened and the mouse habituates to the entire chamber for ten minutes (phase 1). **C)** One strange mouse is placed into a wire cage in one of the side chambers, and the chamber doors are open for ten minutes (phase 2). **D)** A second stranger mouse is placed into a wire cage in the remaining side chambers, and the chamber doors are open for ten minutes (phase 3) (Svedova, 2011).

Before testing began, the cage of subject mice was placed in the testing room for thirty to habituate. During that time period, the two stranger mice (adult, male, wild-type C57BL6 control mice) were placed in wire cages in each chamber side (Figure 2A). After habituation, the stranger mice were placed back into their home cage, and the apparatus was washed with warm water and soap.

The subject mouse was then placed in the center chamber with the chamber doors down for ten minutes in order to habituate. After this period, the thirty-minute three-chamber sociability test began. During the first ten-minute period (phase 1), the side chamber doors were removed, and the mouse was free to roam about the entire chamber (Figure 2B). The time spent in each chamber, defined as over half of the body in the chamber, was recorded to ensure that the mouse did not have a side preference.

Next, the side chamber doors were replaced, and the subject mouse was placed in the middle chamber. A stranger mouse was randomly assigned to one side and placed under the wire cage. The side chamber doors were removed, indicating the start of phase 2 (Figure 2C). The purpose of phase 2 was to quantify preference for social interaction. The time spent in the chamber with the stranger mouse, the target chamber, was compared to the time spent in the empty chamber. After ten minutes had passed, the side chamber doors were again replaced, and the subject mouse was placed in the center chamber. The second stranger mouse was placed in the remaining chamber under the wire cage and cement block (Figure 2D). The side chamber doors were removed. This ten-minute third phase tested the subject mouse's preference for social novelty. The amount of time spent in the chamber with the novel stranger mouse (target chamber) was compared to the amount of time spent with the "old" stranger mouse.

Phases 1, 2, and 3 were video recorded. The time spent in each chamber was manually recorded by two scorers, one blinded and one unblinded, and the average was used. The ratio of time spent in the target chamber to the empty chamber was calculated for each phase and used for data analysis.

The three-chamber sociability test also provided a method to determine the social approach behaviors of the mice. Total frontal contact time was measured during phase 3. Frontal contact time was determined to be any contact by the front half of the subject mouse with the stranger mouse or cage. The behavior typically depicted itself when the stranger mouse had his paws or face against the wire cage. Frontal contact was measured because the mouse could have been in the target chamber but not have been socializing. One blinded scorer measured total frontal contact time.

#### *Self-Grooming test*

Before testing began, the home cage was placed in the testing room with the filter paper off for thirty minutes to habituate, in the same manner as the three-chamber test. Next, the mouse was placed in a clean, empty, transparent mouse cage (7.5"x11.5"x5") for ten minutes to habituate (McFarlane et al., 2008). The mouse was then video recorded while in the single-chamber apparatus for the next ten minutes. The time spent spontaneously grooming during the second ten-minute time period was recorded by two scorers, one of which was blinded. The scores were then averaged. Grooming was determined according to the same criteria used in the three-chamber sociability test. The single-chamber test occurred on a different day than the three-chamber sociability test.

### *Social Transmission of Food Preference*

Social transmission of food preference is a task that has been used to measure communication skills in rats and mice (Galef & Stein, 1985; Wrenn et al., 2003).

Mice were tested in groups of two to five. Twenty-four hours prior to testing, mice were habituated to eating powdered food from jars. The cellulose, heptanoic, and octanoic pellets were crushed so that dietary treatment would be maintained. The jars (2" diameter, 1.5" height) had rounded bottoms and were made of glass. A small metal disk with holes was placed on top of the food. A metal cover with a circular opening was placed on top of the disk to secure its placement. The overall top to the jars was used to prevent the mice from burrowing into and dispersing the food. After habituation, one cagemate (the demonstrator mouse) was placed in a separate cage and given water for 18 hours. During this time period, all mice fasted. At 18 hours, the demonstrator mouse was given one jar of powdered, flavored food (trained flavor). The flavored food was made using regular pellet chow. After two hours, the amount of food the demonstrator mouse had eaten was measured. If it had eaten at least 0.5 grams of the flavored food, the mouse was placed back into its home cage. If it had not, it remained in isolation for hour-long intervals until it had eaten at least 0.5 grams, at which point the mouse was reunited with its cagemates. After thirty minutes of interaction in the home cage, the observer mice were placed into separate cages and given the option of two flavored foods, the trained flavor and a novel or untrained flavor. Again, the flavored food was made with regular pellet chow. After two hours, the mice were placed back into their home cage. Jars were weighed before and after the presentation of food to determine the amount that was eaten. Flavor pairs were 2% cocoa against 1% cinnamon and 1% cinnamon against 0.25% cumin (Ruskin et al., 2013).

STFP is believed to measure the ability of the observers to detect olfactory cues from the demonstrator mouse (Wrenn et al., 2003). Healthy mice engage in social transmission during the thirty minutes and choose to eat the trained flavor because they have recognized the smell from the demonstrator mouse. Mice showing autistic symptoms do not engage in the communication behavior necessary for STFP to successfully take place, thus showing no preference for either the trained or untrained flavor.

### *Rotarod testing*

Rotarod testing assesses balance and motor coordination (Crawley, 2008; Moy et al., 2004). The mouse is placed on a cylinder, which rotates and requires the mice to walk steadily forward. If they do not, they will fall off of the cylinder. On the first day, mice were trained to use the rotarod. They were placed on the rotarod for five minutes at a constant 5 revolutions per minute (rpm). Thirty minutes later, the training session was repeated. On the day of testing, mice were placed on the rotarod for five minutes at 5 rpm, gradually increasing to 40 rpm. Four trials were conducted, with thirty-minute breaks in between each trial. The latency to fall from the rotarod was recorded after each trial. The times recorded were collapsed across the four trials, and these values were used for data analysis.

### *Statistical Testing*

Outliers were removed if they were more than two standard deviations away from the mean. If an outlier was present in the weight, ketone, glucose, or food intake levels at five or eight weeks, that test subject was removed from all analyses, including behavioral testing. Less than 5% and 6% of the subjects' data were eliminated from weight and glucose data analysis and individual behavioral test analysis, respectively. When the pellet and cellulose groups did not differ, the heptanoic and octanoic treatment groups were only compared to the cellulose control.

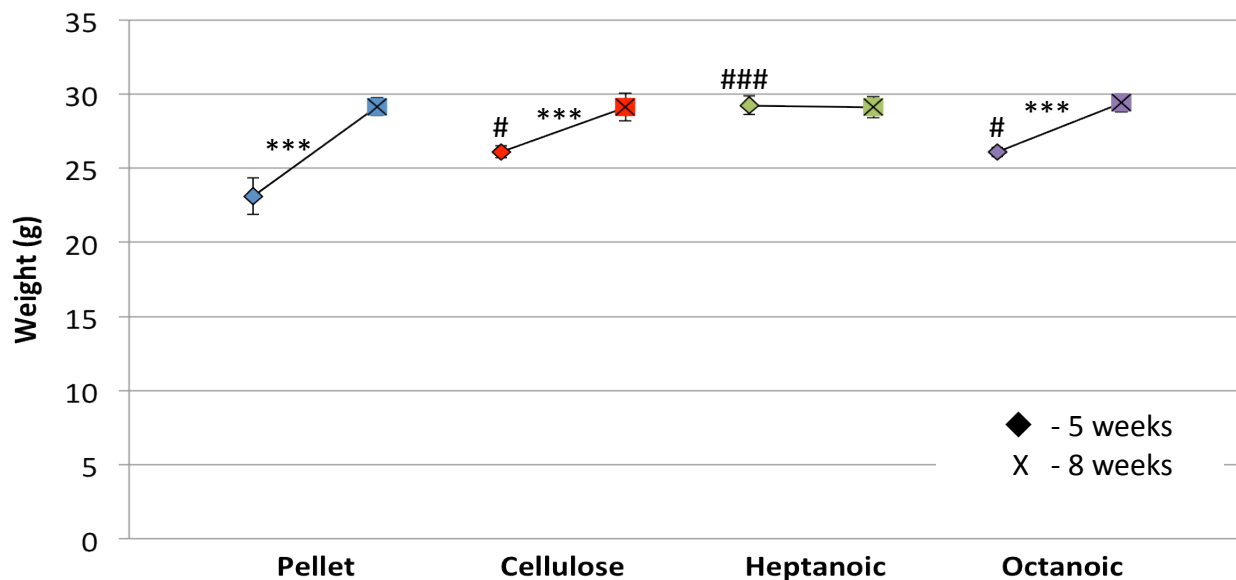
Otherwise, they were compared to the pellet control. One-way or two-way repeated measures ANOVA was used for analysis, as appropriate. Post-hoc tests used were Tukey, for all pairwise comparisons, and Bonferroni, for all comparisons to the pellet control or cellulose group. ANOVA on ranks was used for the rotarod test. All data are presented as the mean ( $\pm$  SEM).

## RESULTS

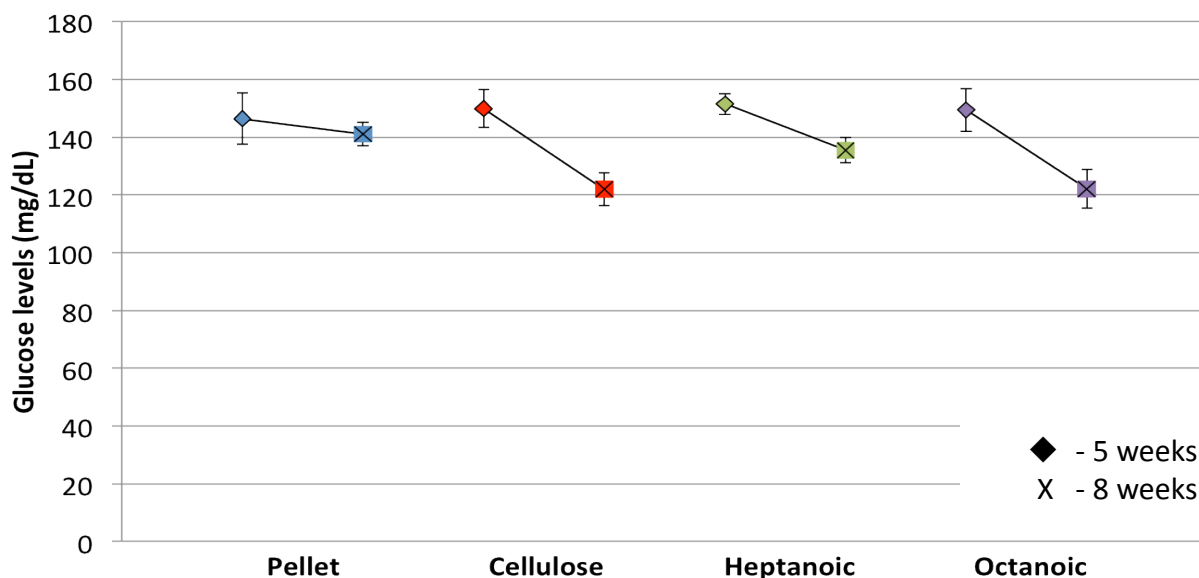
### *Weight and Blood*

Body weight, glucose levels, and  $\beta$ HB levels of each mouse were measured at five weeks and eight weeks. We found an unexpected difference with regard to baseline weights, with the pellet control group having a significantly lower base weight, indicating that there was a poor initial balancing of weights. However, weight increased significantly over the three weeks of treatment in the pellet, cellulose, and octanoic groups. The heptanoic group did not show a significant change in weight over time, but the weights were initially high; the heptanoic group did not differ from the other groups after three weeks of treatment (Figure 3).

Glucose levels were not significantly affected by any particular treatment, and decreased glucose levels were seen among all groups at eight weeks of age (Figure 4). With respect to ketones, the cellulose treatment group showed a significant decrease in  $\beta$ HB levels after three weeks of treatment whereas the octanoic group showed a significant increase as compared to all others. Additionally, the octanoic group had significantly higher  $\beta$ HB levels at eight weeks compared to the pellet control group, indicating that the octanoic group experienced a high level of ketosis (Figure 5).

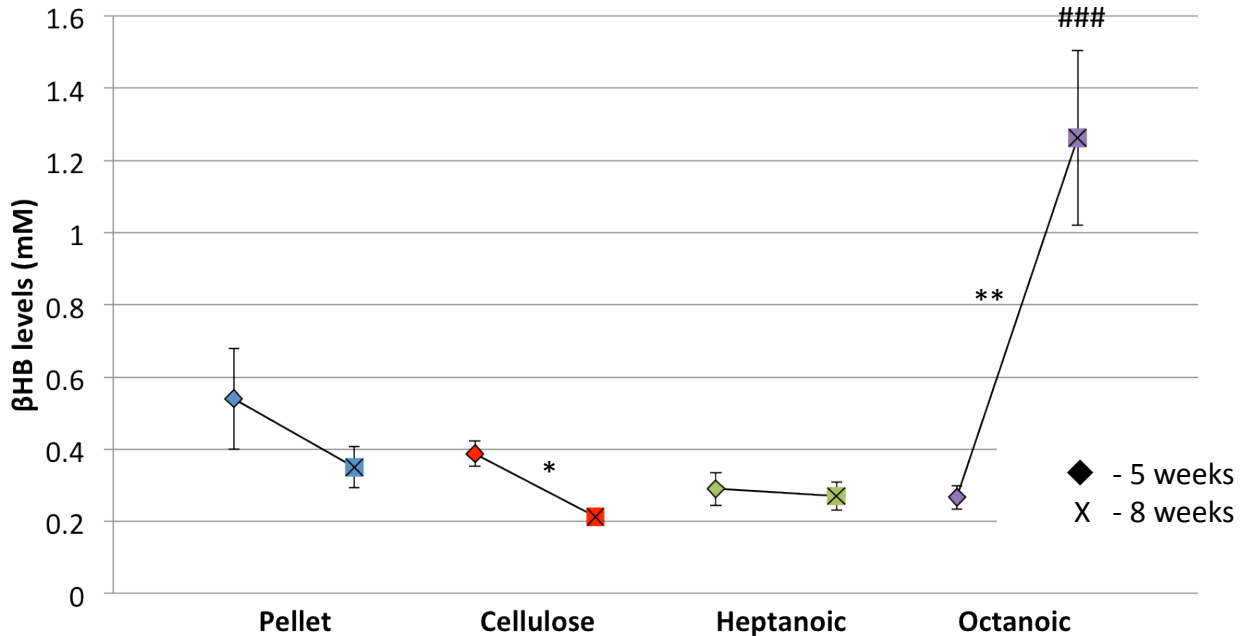


**Figure 3: Average body weights of mice in each treatment group at five weeks and eight weeks of age.** At baseline, weight was significantly lower in the pellet control group. Weight significantly increased over the three weeks of treatment in the pellet, cellulose, and octanoic groups. Factors for ANOVA: treatment  $p = 0.018$ ; time  $p < 0.001$ ; treatment x time  $p < 0.001$ . \*\*\*  $p \leq 0.001$ , Tukey post-hoc compared to five weeks. #  $p \leq 0.05$ , ###  $p \leq 0.001$ , Tukey post-hoc compared to pellet control (pellet:  $n=5$ ; cellulose:  $n=8$ ; heptanoic:  $n=10$ ; octanoic:  $n=8$ ).



**Figure 4: Average glucose levels of mice in each treatment group at five weeks and eight weeks.** Treatment did not have an effect on glucose levels. Factors for ANOVA: treatment  $p = 0.189$ ; time  $p = 0.006$ ; treatment x time  $p = 0.248$  (pellet:  $n=5-8$ ; cellulose:  $n=8$ ; heptanoic:  $n=10$ ; octanoic:  $n=3-8$ ).

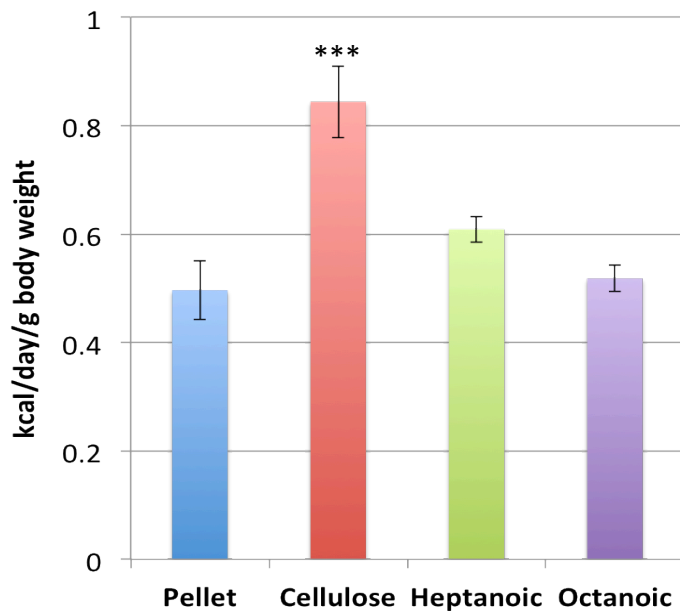




**Figure 5: Average  $\beta$ HB levels of mice in each treatment group at five weeks and eight weeks.** The cellulose treatment group showed a significant decrease in  $\beta$ HB levels from five to eight weeks of age whereas the octanoic group showed a significant increase. Compared to the pellet control treatment group, the octanoic group had significantly higher  $\beta$ HB levels at eight weeks. Factors for ANOVA: treatment  $p < 0.001$ ; time  $p = 0.712$ ; treatment x time  $p = 0.004$ . \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , Tukey post-hoc compared to five weeks. ###  $p \leq 0.001$ , Tukey post-hoc compared to pellet control (pellet:  $n=5-8$ ; cellulose:  $n=8$ ; heptanoic:  $n=10$ ; octanoic:  $n=3-8$ ).

### ***Food Intake***

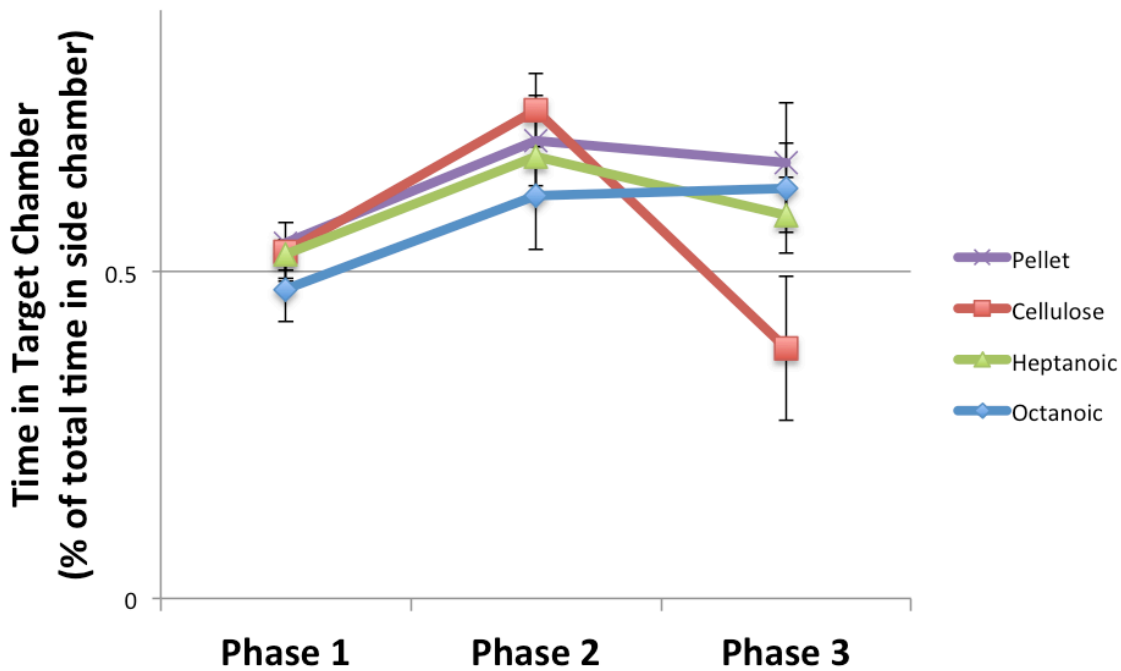
The daily weight of food eaten was measured once a week over the course of treatment, and the data was averaged in order to obtain an estimate of the daily food intake for each mouse. However, because of varying caloric content, interpretation of the raw data must be done cautiously. As such, caloric intake was calculated using the value of metabolizable energy of the standard pellet chow (LabDiet2013) and the net energy value of MCTs (Ingle et al., 1999). Although net energy accounts for heat loss while metabolizable energy does not, the heat loss in mice is fairly small, so the values are highly comparable (L. Tracey, personal communication, March 12, 2014). The mice in the cellulose treatment group had a higher caloric intake per gram of body weight at eight weeks of age compared to the pellet control (Figure 6).



**Figure 6: Average daily caloric intake per g of body weight at eight weeks of age.** Compared to the pellet control, the cellulose treatment group had a higher caloric intake of food during the last week of treatment. Factor for ANOVA: treatment  $p \leq 0.001$ . \*\*\*  $p \leq 0.001$ , Bonferroni t-test compared to pellet control (pellet:  $n=5$ ; cellulose:  $n=8$ ; heptanoic:  $n=10$ ; octanoic:  $n=8$ ).

### *Three-Chamber Sociability*

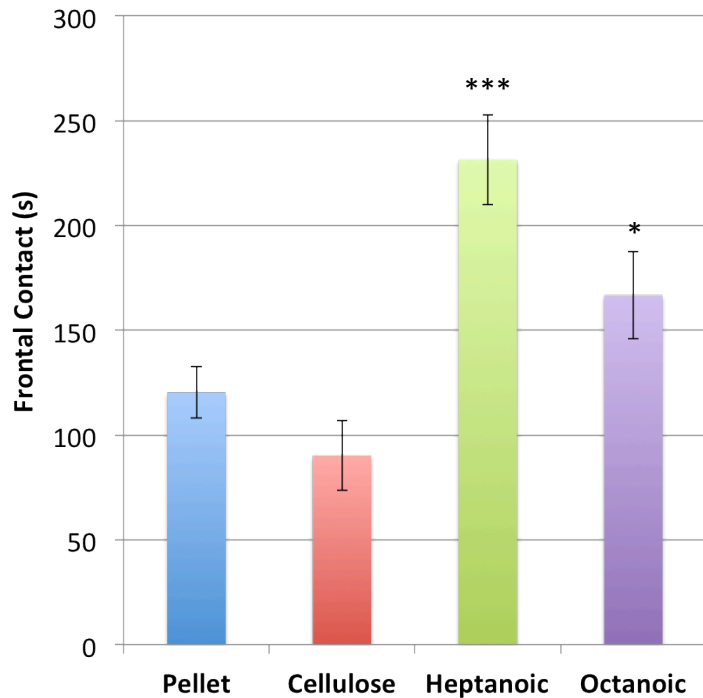
In the three-chamber test for sociability, the time the mouse spent during each of the three phases was measured to check for side bias, preference for social interaction, and preference for social novelty during phases 1, 2, and 3, respectively. The graph illustrates the data as the ratio of time spent in the target chamber compared to the empty chamber (phase 2) or chamber with the old stranger mouse (phase 3). Dietary treatments did not significantly influence sociability. None of the mice showed a side preference during phase 1, and all groups spent more time with the stranger mouse than the empty chamber during phase 2 ( $p = 0.008$ , Figure 7). This was unexpected with the pellet and cellulose control groups.



**Figure 7: Relative sociability of mice as measured by the amount of time spent in a chamber with a stranger mouse compared to an empty chamber (phase 2) and time spent in a chamber with a novel mouse compared to the old stranger mouse (phase 3).** The three-chamber sociability test illustrated that treatment had no effect on sociability. Factors for ANOVA: treatment  $p = 0.587$ ; time  $p = 0.007$ ; treatment x time  $p = 0.19$  (pellet:  $n=8$ ; cellulose:  $n=8$ ; heptanoic:  $n=9$ ; octanoic:  $n=8$ ).

### ***Social Approach***

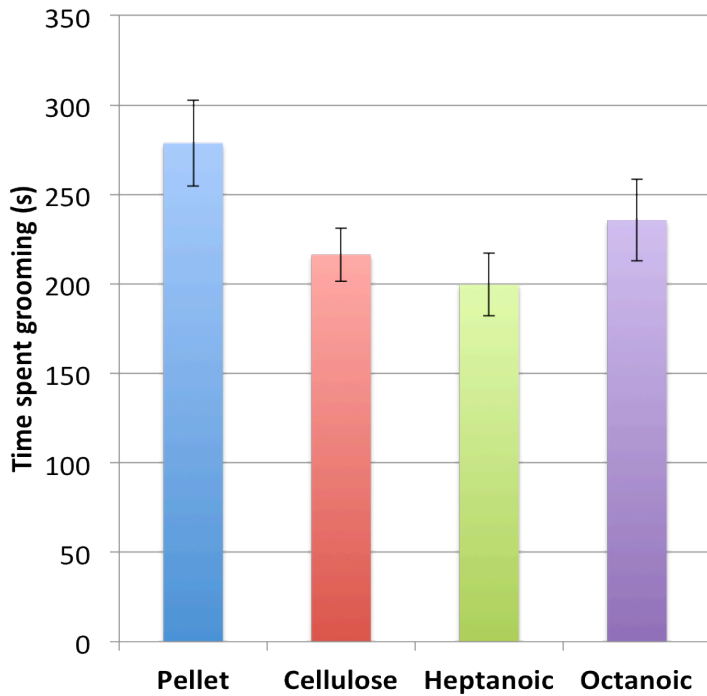
In the three-chamber test, the total amount of time the mouse spent partaking in frontal contact with either the novel or old stranger mouse during phase 3 was used to determine if the mouse engaged in social approach. Compared to the cellulose control, the heptanoic and octanoic treatment groups spent significantly more time engaging in social approach behaviors (Figure 8).



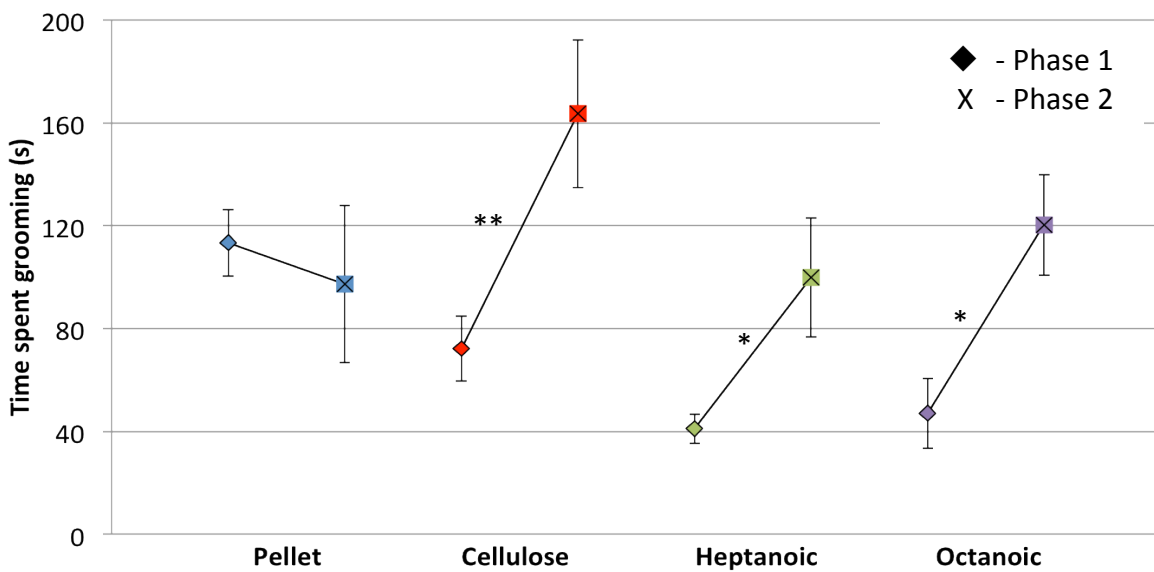
**Figure 8: Total amount of time spent engaging in frontal contact during phase 3 of the three-chamber sociability test.** Compared to the cellulose group, the heptanoic and octanoic treatment groups spent significantly more time engaging in frontal contact during phase 3. Factor for ANOVA: treatment  $p \leq 0.001$ . \* $p \leq 0.05$ , \*\*\*  $p \leq 0.001$  Bonferroni t-test compared to cellulose control (pellet:  $n=7$ ; cellulose:  $n=8$ ; heptanoic:  $n=9$ ; octanoic:  $n=8$ ).

### ***Self-Directed Repetitive Behavior***

The amount of time spent grooming during the single-chamber test and phases 1 and 2 of the three-chamber test were used as an indicator of self-directed repetitive behavior. Compared to the cellulose group in the single-chamber grooming task, the heptanoic and octanoic treatment had no effect on self-directed repetitive behavior (Figure 9). During the three-chamber task, the cellulose, heptanoic, and octanoic treatment groups spent significantly more time grooming in phase 2, when the stranger mouse was present, than in phase 1. There were no significant differences in the time spent grooming in the pellet treatment group or between experimental groups during the three-chamber test (Figure 10).



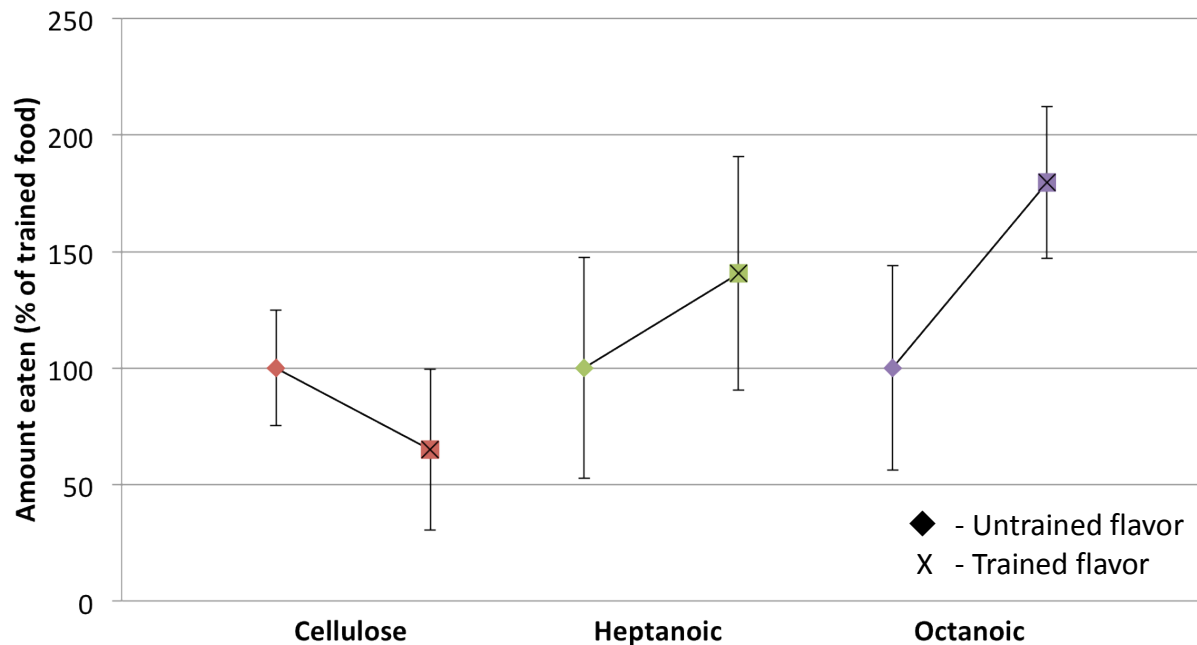
**Figure 9: Amount of time spent grooming during the single-chamber grooming task.** Treatment had no effect on self-directed repetitive behaviors compared to the cellulose group. Factor for ANOVA: treatment  $p = 0.048$ . Bonferroni t-test compared to cellulose (pellet:  $n=8$ ; cellulose:  $n=8$ ; heptanoic:  $n=10$ ; octanoic:  $n=8$ ).



**Figure 10: Amount of time spent grooming during phase 1 compared to phase 2 as a measure of repetitive behaviors.** The cellulose, heptanoic, and octanoic treatment groups spent significantly more time grooming in phase 2 (presence of one stranger mouse) than in phase 1. Factors for ANOVA: treatment  $p = 0.106$ ; time  $p < 0.001$ ; treatment x time  $p = 0.053$ . \*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$ , Tukey post-hoc compared to phase 1 (pellet:  $n=8$ ; cellulose:  $n=8$ ; heptanoic:  $n=9$ ; octanoic:  $n=8$ ).

### *Social Transmission of Food Preference (STFP)*

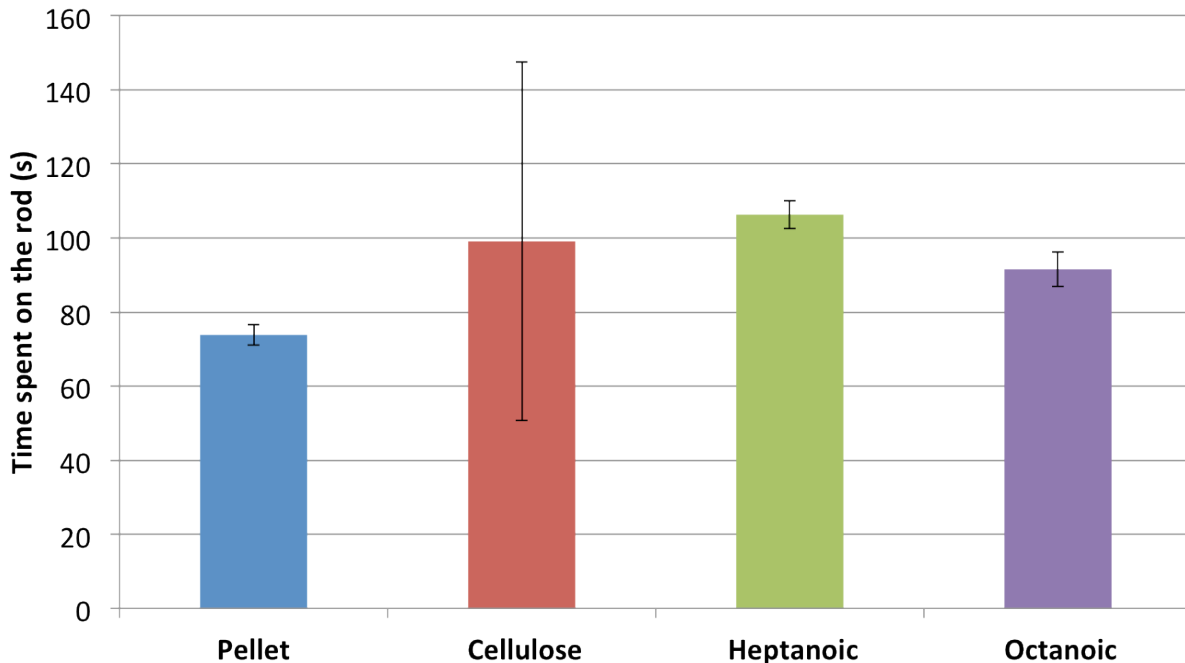
STFP was used as a relative measure of communication via olfactory cues. STFP was not included for the pellet treatment due to technical errors. No significant differences were found within or between experimental groups in the STFP test (Figure 11).



**Figure 11: Relative ability to communicate as demonstrated by the STFP test.** No significant differences were found in the STFP test. Mice in the pellet control group were not tested. Factor for ANOVA: treatment  $p = 0.927$ ; flavor  $p = 0.689$ ; treatment x flavor  $p = 0.499$  (cellulose:  $n=6$ ; heptanoic:  $n=7$ ; octanoic:  $n=6$ ).

### *Rotarod*

The rotarod was used to test motor control and coordination. No significant differences were found among or between experimental groups in the rotarod test (Figure 12). For statistical purposes, the four trials were collapsed over time per mouse, and those values were used.



**Figure 12: Motor control as demonstrated by the rotarod test.** Treatment had no effect on the amount of time the mice spent on the rotarod. Factor for ANOVA on ranks: treatment  $p = 0.065$  (pellet:  $n=7$ ; cellulose:  $n=2$ ; heptanoic:  $n=3$ ; octanoic:  $n=3$ ).

## DISCUSSION

The major goal of this study was to determine the efficacy of MCTs and the role of anaplerosis in establishing ketosis and alleviating autistic symptoms in mice. In order to carry this out, BTBR mice were randomly assigned to a pellet, cellulose, heptanoic, or octanoic treatment group. Body weight, glucose levels and  $\beta$ HB, and food intake were measured over the course of treatment. Behavioral testing was conducted in order to quantify sociability and social approach, repetitive behavior, communication, and motor coordination skills. The results from the laboratory behavioral tests produced conflicting data regarding the efficacy of MCTs on alleviating autistic symptoms. However, the MCT KDs increased ketones and trended towards alleviating autistic symptoms, suggesting its mechanistic basis may be similar to that of the LCT KD. Furthermore, a MCT KD is clinically relevant, and as such, should be investigated further.

## ***Rationale***

Previous work has illustrated that a strict LCT KD (6.6:1 fats to proteins and carbohydrates) alleviates autistic symptoms in the BTBR mouse model (Ruskin et al., 2013) whereas a moderate and clinically relevant LCT KD was ineffective (Reuman, 2013). This work suggests that the increase in ketone bodies due to the KD has an effect on the CNS that consequentially results in the beneficial alleviation of autistic symptoms. Because MCTs are hydrolyzed faster and more efficiently than LCTs (Labarthe et al., 2008), a lower ratio of fats to proteins and carbohydrates is allotted, allowing a MCT KD to be clinically relevant, while inducing the same effect as a LCT KD (Liu & Wang, 2013). Thus, it was hypothesized that a MCT KD would induce a level of ketosis similar to that of the strict LCT KD because MCTs have been shown to cause an increase in ketone bodies, ATP, and acetyl CoA (de Baulny & Superti-Furga, 2006). It was further hypothesized that odd-numbered MCTs would be more effective at alleviating autistic symptoms because they undergo anaplerosis, whereas even-numbered MCTs do not (Borges & Sonnewald, 2012). This suggests that an increase in ATP and the subsequent increase in adenosine may also be a possible mechanism by which the KD acts.

## ***Pellet and Cellulose Control Groups***

Initially, the intended control group for this study was a standard chow control diet balanced with 17% cellulose. However, preliminary data analysis indicated that the cellulose group did not display autistic symptoms, which is expected of the BTBR mouse strain (McFarlane et al., 2008; Silverman, Yang, & Crawley, 2010). As a result, a group of mice solely on the standard chow diet was added as another control. If there was no difference between the pellet and cellulose groups, the cellulose group was used as the control in data analysis. The data



regarding both the pellet control and cellulose groups for this study has been mostly inconsistent with other studies, namely in the three-chamber test for sociability and repetitive behavior. As such, all data must be regarded carefully. Future experiments might benefit from baseline behavioral tests conducted before the administration of the diet to confirm autistic behaviors before treatment.

### ***Weight and Blood***

Body weight and blood glucose and ketone levels were measured before treatment began and at eight weeks of age. Body weight was measured in order to ensure that the diet did not affect mouse growth and development or alter weight gain. In order to determine if the mice were in ketosis, blood glucose and ketone levels were measured. Typically, the ketogenic diet results in increased levels of ketones and decreased levels of glucose (Masino et al., 2013).

Unfortunately, the initial weights between the four treatment groups were not balanced well; compared to the pellet control, the cellulose, heptanoic, and octanoic groups had significantly greater weights at five weeks of age before treatment began. Nonetheless, the pellet, cellulose, and octanoic groups showed a significant increase in weight at eight weeks of age compared to five weeks. Only the mice in the heptanoic treatment group did not increase in weight, although the weight at eight weeks was not different. If weight were expressed as the percent of baseline, the heptanoic treatment group would not show any growth. However, the average weights of all three treatment groups at eight weeks were similar. Furthermore, all mice were in the weight range for their age group (Reuman, 2013). Because of the baseline weight discrepancy, it is difficult to make any interpretations regarding weight and growth.

Glucose levels within any of the four treatment groups did not significantly change over the course of treatment. In light of the risk of hypoglycemia associated with the LCT KD, the

lack of significant difference of glucose levels is a promising aspect of the MCT KDs, indicating that fewer medical risks may be associated with them.

Blood  $\beta$ HB levels suggest that the octanoic treatment group experienced a high level of ketosis.  $\beta$ HB levels significantly increased in the octanoic treatment group and significantly decreased in the cellulose group from five weeks of age to eight weeks. Because octanoic acid promotes ketosis and bypasses glycolysis, the result was expected. However, no difference was observed in the heptanoic treatment group although the same logic applies. The lack of a measured effect of ketosis in the heptanoic group is most likely due to the fact that the Precision Xtra Ketone Meter and Strips undergo a timed reaction between  $\beta$ HB dehydrogenase (received from the blood sample) and NAD (in the strips) to produce NADH. The influx of electrons causes an electrical change that then produces a detectable signal the meter can report. However, because heptanoate is an odd-numbered MCT, the reaction that occurs uses  $\beta$ -hydroxy pentanoic acid (which is produced from C5 ketone bodies) instead of  $\beta$ HB. The reaction with  $\beta$ HP is approximately twenty times slower than that of the  $\beta$ HB reaction (Bergmeyer et al., 1967). As such, the reaction would not be near completion by the time the meter reports the ketone levels, which is probably why no change was observed regarding ketone levels in the heptanoic treatment group. However, it can be reasonably assumed that the mice in the heptanoic treatment group were in ketosis as heptanoate molecules can be broken down into C5 ketone bodies (Borges & Sonnewald, 2012). Future studies should consider the inaccuracy in using the Precision Xtra Meter to measure C5 ketones and utilize a different method.

### ***Food Intake***

The caloric intake of each treatment group is important in order to determine the palatability of the diets and the relative impact the amount of food eaten may have had on

altering the metabolism of the mice. When considering the caloric intake of the groups, only the cellulose group had a significantly higher caloric intake than the pellet group, suggesting that the heptanoic and octanoic treatment would not have affected growth. Furthermore, high levels of fiber within the cellulose diet may have impeded digestion or made it more metabolically costly, resulting in a higher caloric intake by the mice within that treatment group.

It is important to note two limitations. First of all, the pellets were placed in a wire cage above the mice. Some food may have fallen out or have been lost in the bedding, impacting the results obtained. Furthermore, the mice were housed socially, and the amount of food eaten over the course of a day was calculated and divided by the number of mice in that particular cage. Although we obtained an estimate, there is no specific data regarding the exact amount of food each animal consumed.

### ***Sociability and Social Approach***

The three-chamber test is used to quantify sociability and social approach. Impairments in social behaviors are characteristic of autism. In other studies, BTBR mice have shown decreased levels of sociability, as measured by the time spent in a chamber with a stranger mouse compared to an empty chamber (phase 2: preference for social interactions) and by the time spent in a chamber with a novel mouse compared to the first stranger mouse (phase 3: preference for social novelty) (McFarlane et al., 2008; Ruskin et al., 2013; Silverman et al., 2010). However, the results from the sociability test were unusual. No significant effect of treatment on sociability was illustrated. Rather, the time the pellet control spent in the target chamber during phase 2 was unusually high with a ratio of approximately 0.7. In a study that used BTBR mice and the same chow diet, the ratio of time spent in the target chamber during

phase 2 was approximately 0.55 (Ruskin et al., 2013). Because the pellet control did not behave as expected, no interpretation is available for the effect of treatment on sociability.

The three-chamber test also provided a measured social approach, which did not conform to the results of sociability. No difference existed between the pellet and cellulose groups, so the cellulose group was used as the control. Compared to the cellulose control, the heptanoic and octanoic treatment groups spent significantly more time engaged in frontal contact, which is what we expected. The amount of time the pellet and cellulose controls spent engaged in pellet control was approximately the same as reported by Ruskin et al. (2013), illustrating that the consistency and reliability of this test has been maintained and may be more indicative of social behavior than the sociability measurement of the three-chamber test. Both the heptanoic and octanoic treatment groups were in ketosis (or expected to be: see above) and showed a significant increase in social approach behaviors, suggesting that a MCT KD has the potential to alleviate autistic symptoms.

### ***Self-Directed Repetitive Behavior***

Self-grooming is a measure of self-directed repetitive behavior, which is characteristic of individuals with autism. Repetitive behavior was measured in mice in a single-chamber self-grooming test as well as during phase 1 and 2 of the three-chamber test. Because no difference existed between the pellet and cellulose groups, the cellulose group was used as the control. Compared to the cellulose control, treatment had no effect on self-directed repetitive behaviors. These results are not easy to interpret clearly; for now they should be considered with care.

The grooming behavior during the three-chamber task is unclear. The cellulose, heptanoic, and octanoic treatment groups all significantly groomed more in phase 2 compared to phase 1. Furthermore, the time spent grooming in the pellet control group trended downward

when it was expected to trend upwards in the presence of a stranger mouse. In Ruskin et al., (2013) BTBR mice on the pellet control diet spent significantly more time grooming in phase 2 compared to phase 1. Specifically, the mice spent approximately 50 s grooming during phase 1 and approximately 150 s grooming in phase 2. In this study, the mice spent approximately 110 s grooming in phase 1 and 90 s grooming in phase 2. The inconsistency in results between studies suggests that the results, again, should be considered carefully.

As it stands, the data suggests that the MCT KDs do not have an effect on alleviating self-directed repetitive behavior. However, the lack of a reliable control makes the interpretation difficult.

### ***Communication***

The social transmission of food preference task has been used to measure communication skills in rats and mice (Galef & Stein, 1985; Wrenn et al., 2003). BTBR mice have shown impairments in communication as illustrated by the STFP test, which is a behavioral characteristic of people with autism (McFarlane et al., 2008). One shortcoming is that pellet control group was not included due to technical errors. However, the cellulose group did not exhibit STFP, suggesting that it may be a reliable control for this test. It was expected that the heptanoic and octanoic treatment groups would eat significantly more of the trained flavor compared to the untrained flavor; however this was not the case. Within each treatment group, no significant difference was observed, most likely due to the high standard error and small sample size.

### ***Motor Control***

Because those with ASD have been shown to have significant motor coordination defects (Fournier et al., 2010), the rotarod test was used as a measure of motor control in the BTBR mice.

Treatment had no effect on the time spent on the rotarod. Although there was a trend for a positive effect in the heptanoic treatment group, the results are unclear because the sample size of each group was relatively low, with one group having a sample size of two and two groups having a sample size of three. More data needs to be collected before any interpretations can be made, but the rotarod test does not seem to be impaired by the MCT KDs tested here.

## **SUMMARY & CONCLUSION**

Overall, we found the MCT KD increased ketones, based on the octanoic group. However, our behavioral findings were mixed. The mice in the pellet and cellulose groups did not clearly exhibit autistic behaviors as expected, primarily in the three-chamber test for sociability and repetitive behaviors. Furthermore, baseline weights were not balanced,  $\beta$ HB levels were not measured accurately in the heptanoic treatment group, the pellet control group was not included in analyses for deficits in communication, and the rotarod test had a very small sample size. More work could resolve these issues. However, it is important to note that the pellet and cellulose control mice did portray autistic behaviors in regards to the frontal contact task, and the heptanoic and octanoic treatment groups spent significantly more time engaged in frontal contact than the cellulose control, suggesting that the MCT KDs do have the potential to alleviate autistic symptoms. Due to the aforementioned inconsistencies, it is difficult to draw meaningful global interpretations, and all test-specific interpretations must be drawn cautiously. The current study should be investigated further and expanded in order to understand the impact of this metabolic treatment on autistic symptoms as well as the mechanism by which the KD works.

In conclusion, the results of the current study were conflicting and did not permit clear interpretations. More data and a reliable control group are needed to establish a compelling case for this treatment. Because a MCT KD metabolic treatment allows for a greater allowance of proteins and carbohydrates, it is important to continue this investigation in hopes of becoming a viable therapy for patients with autism.

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