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# Effect Of The Ketogenic Diet On Seizures In The Methionine Sulfoximine Model Of Mesial Temporal Lobe Epilepsy

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Effect of the Ketogenic Diet on Seizures in the Methionine Sulfoximine Model of Mesial  
Temporal Lobe Epilepsy

A thesis submitted to the  
Yale University School of Medicine  
in Partial Fulfillment of the Requirements of the  
Degree of Doctor of Medicine

By Clayton Haldeman

2013

## Abstract

### Effect of the Ketogenic Diet on Seizures in the Methionine Sulfoximine Model of Mesial Temporal Lobe Epilepsy

Clayton Haldeman

2013

About two-thirds of patients with one of the most common forms of epilepsy – mesial temporal lobe epilepsy (MTLE) – cannot control their seizures with current antiepileptic drugs. More efficacious therapies for this disorder are therefore needed. A low carbohydrate, high fat – i.e. ketogenic – diet (KD) is a highly effective treatment for many types of epilepsies; however, whether the diet is effective in reducing seizures in MTLE is relatively unexplored. As a first step towards understanding the role of the KD in MTLE, we investigated whether the diet reduces the frequency and severity of seizures in a novel rat model of this disorder.

Thirty-eight male Sprague Dawley rats were implanted with an osmotic pump injecting the glutamine synthetase inhibitor methionine sulfoximine (MSO) into the polymorphic layer of the right hippocampus. Beginning immediately after surgery, the rats were continuously monitored with intracranial EEG and simultaneous video recordings. One week post-surgery, rats were assigned to either a KD with 20% caloric restriction or a standard chow (SC) fed ad libitum and further monitored for another two to four weeks. EEG analysis was correlated with video recordings to determine the frequency and severity of seizures according to a modified Racine scale.

There was no significant difference in the weekly frequency of seizures in rats treated with the KD versus SC. Neither was there any difference in the severity of seizures between rats treated with the KD and rats fed SC. These data should be interpreted with caution because animal models of MTLE may respond differently to the KD than humans with the disorder. Also, a lack of significant difference does not rule out the presence of a real, but small difference that cannot be detected due to the statistical power of the present study. Nevertheless, the data suggest that the KD may not be an effective treatment for MTLE.

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## **Chapter 1: Introduction**

There are approximately 1.6 million people in the United States suffering from epilepsy<sup>1</sup>. While most patients with epilepsy maintain good control over their disease, temporal lobe epilepsy (TLE) is often drug resistant with upwards of 40% of patients continuing to have seizures despite treatment with appropriate anti-epileptic drugs (AEDs)<sup>2,3</sup>. Uncontrolled seizures greatly impact patient's quality of life with significant psychological, economical, and social impact<sup>4</sup>. Furthermore, in animal studies, recurrent seizures have been shown to lead to cell loss and synaptic reorganization, causing permanent brain injury<sup>5</sup>. Thus, there is a need for greater understanding of the pathophysiology of TLE and a necessity to develop more efficacious therapies for this disease.

One possible therapy for combating medically intractable seizures, which has regained popularity recently, is the ketogenic diet (KD). The KD was originally developed in the 1920s, when relatively few antiepileptic medications were available, to imitate the metabolic effects of starvation after observations that fasting decreased seizure frequency<sup>6</sup>. The KD is a high-fat, low carbohydrate diet that improves the brain energy stores in patients with epilepsy<sup>7</sup>, reduces the frequency of spontaneous recurrent seizures in many types of this disease, particularly pediatric epilepsies<sup>8</sup>, and protects against neuronal loss in animal models of TLE<sup>9</sup>. Though a number of different mechanisms of action of seizure reduction have been proposed for the diet, there is no consensus currently which hypothesis is correct. Moreover, whether the KD is an effective and sustainable long-term treatment for patients with MTLE has not been fully established. The goal of this thesis is to investigate whether the KD reduces the frequency and

severity of seizures in an animal model of MTLE. Such information would address the lack of knowledge on the diet's efficacy in MTLE, and could potentially provide us with an animal model for studies on the mechanisms of the KD in this disorder.

## **1.1 Epilepsy Review**

Epilepsy is a chronic neurologic condition affecting nearly 50 million people worldwide<sup>10</sup>. Epilepsy is not a single disease but a variety of disorders reflecting underlying brain dysfunction characterized by recurrent seizures. Seizures are defined by The International League Against Epilepsy (ILAE) as “a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neural activity in the brain.”<sup>11</sup> Most seizures can be broadly classified into one of two types: partial (localization-related) or generalized<sup>12</sup>.

Generalized seizures are abnormal synchronous electrical activity that involves both hemispheres at onset and consciousness is lost from the start. Just as with partial seizures, generalized seizures are classified primarily based on clinical findings<sup>13</sup>. For example, during absence seizures the patient abruptly loses consciousness and may appear to stare blankly for a short period of time, then suddenly regain consciousness with no residual deficits. Generalized tonic-clonic seizures are characterized by prolonged contraction of skeletal muscle during the tonic phase, followed by rapid alternation of muscle relaxation and contraction during the clonic phase.

Partial seizures, also known as localization-related or focal seizures, are abnormal bursting electrical activity that occurs in a localized region of the brain. Partial seizures can be further broken down into simple partial and complex partial seizures. Simple



partial seizures spare consciousness, while complex partial seizures result in loss of consciousness. Partial seizures may begin with localized motor, sensory, autonomic or psychic symptoms. Partial seizures can often times be preceded by an aura, or premonition, that patients can recognize as a forewarning to an impending seizure. When simple or complex partial seizures spread to involve the entire brain, they are termed secondarily generalized seizures. Secondarily generalized seizures are often tonic-clonic seizures that begin with posturing or head and eye deviation.

TLE is a particularly devastating type of focal epilepsy characterized by spontaneous, progressive partial seizures originating in the temporal lobe. The seizures may or may not secondarily generalize. The estimates of the prevalence of TLE vary<sup>14</sup>. In an epidemiological study in Rochester, MN, the incidence rate of TLE was 10.4 per 100,000 between 1945 and 1964, and 6.5 between 1935 and 1944<sup>15</sup>. While many of these patients maintain good control of their seizures with medication, up to 40% of patients with TLE are resistant to anti-epileptic medications<sup>3</sup>.

Several schemes have been proposed for further classification of TLE. One of these schemes divides the disorder into two types based on the anatomic origin of the seizure focus: Seizures that originate in the medio-basal (limbic) temporal structures, mesial temporal lobe epilepsy (MTLE), and seizures that originate in other (usually lateral) parts of the temporal lobe. The hippocampus is located in the medial temporal lobe, and it is commonly regarded as the site of origin for MTLE, and surgical removal of the hippocampus often results in good control of seizures in these patients<sup>16</sup>. Pathological examination of the resected hippocampus in many of these patients exhibit a characteristic pattern of pathological changes, notably atrophy, induration, glial

proliferation, and preferential loss of neurons in CA1, CA3, and the dentate hilus.<sup>17</sup>

Mossy fiber sprouting and selective loss of somatostatin and neuropeptide Y-containing hilar neurons can also be seen<sup>18,19</sup>. All these changes are collectively known as hippocampal sclerosis (HS).

For patients with MTLE, the first episode of seizures typically begins during the first 15 years of life. Some patients initially respond well to AEDs, and their seizures can be controlled with medications for a time. Typically, however, seizures return around adolescence or early adulthood. Auras are frequent, with visceral auras being the most common type. Complex partial seizures are the most frequent type of seizures in these patients, with generalized tonic-clonic seizures occurring occasionally<sup>20</sup>.

What causes MTLE to develop is currently unknown. However, there is an association between the development of MTLE and the presence of early risk factors such as prolonged childhood febrile seizures, trauma, or intracranial infection<sup>21,22</sup>. There appears to be a progressive aspect to MTLE as well, that once set in motion, the disorder continues to evolve over time<sup>20</sup>. Despite the fact that multiple new AEDs have come on the market in recent years, many patients remain refractory to medications. There is a need for more effective therapies with fewer side effects. It is important to understand the underlying mechanism of MTLE in order to develop improved therapies.

## **1.2 Possible Mechanisms of MTLE**

Despite the fact that focal seizures are the most common type of seizures, the underlying mechanism of seizure generation remains poorly understood. Much of our current knowledge on MTLE comes from human studies using scalp and intracranial

EEG recordings, brain microdialysis, neuroimaging, and pathological investigations of surgically resected brain tissue. Many investigations also involve various animal models of MTLE.

### **1.2a Glutamate Hypothesis**

Glutamate is the most abundant excitatory neurotransmitter in the brain.

Glutamate concentrations in plasma are 50-100 micromol/L but only 0.5-2 micromol/L in the brain extracellular fluid (ECF)<sup>23</sup>. The low ECF concentrations, which are essential for optimal brain function, are maintained by neurons, astrocytes, and the blood-brain barrier (BBB). Glutamate does not readily cross the BBB and therefore the majority of glutamate is synthesized from blood-derived glucose and transamination of alpha-ketoglutarate<sup>24</sup>.

An excess of extracellular glutamate in the hippocampus may be one of the key molecular causes of seizures and brain damage in MTLE. In *in vivo* microdialysis studies have demonstrated that interictal extracellular glutamate is elevated five-fold more in the epileptogenic vs. the nonepileptogenic human hippocampus<sup>25</sup>. Unexpectedly, in those patients with hippocampal sclerosis, interictal extracellular glutamate concentrations are considerably higher than in patients without this pathology, even though patients with sclerosis have lost up to 80% of neuronal density<sup>26-28</sup>. Extracellular hippocampal glutamate has also been shown to increase six-fold above the interictal level during a seizure and remain elevated for at least 20 minutes after cessation of seizure activity, suggesting a role for glutamate in triggering seizures<sup>29</sup>. Intraoperative isotopic tracer (<sup>13</sup>C) studies suggest that the accumulation and impaired clearance of glutamate in MTLE is due to slowing of the glutamate–glutamine cycle metabolism in the sclerotic and epileptogenic hippocampus compared with the nonsclerotic and nonepileptogenic

hippocampus or the normal occipital neocortex<sup>30</sup>. Finally, oral and subcutaneous administration of glutamate and its analogues has been shown to cause seizures in rhesus monkeys, cats, and mice.<sup>31</sup> All these findings taken together suggest that glutamate is a crucial element in the pathophysiology of this disease.

Dysfunction of glutamine synthetase (GS) in astrocytes has also been hypothesized to cause an excess of extracellular brain glutamate. GS is necessary for the metabolism of glutamate to glutamine, and the enzyme is deficient in discrete areas of surgically resected, epileptogenic hippocampal formations from patients with MTLE. Eid et al demonstrated a 35-40% loss of GS protein and activity in astrocytes in the sclerotic hippocampus when compared to cadaver hippocampi or patients with MTLE and no HS<sup>32</sup>. It was postulated that the deficiency in GS could slow the conversion of glutamate to glutamine and lead to a buildup of astrocytic and extracellular glutamate. However, the lack of GS in patients with MTLE does not necessarily mean that a deficiency in GS causes seizures, and animal studies are required to address this issue. Notably, infusion of the GS inhibitor, methionine sulfoximine (MSO) into the hippocampus in rats, results in recurrent seizures<sup>33</sup>. Moreover, MSO-treated rats sometimes exhibit neuropathological changes similar to HS, such as unilateral atrophy of the hippocampus with loss of neurons in the CA1<sup>34</sup>. Taken together, these findings implicate a loss of GS in the causation of MTLE.

### **1.2b Role of Astrocytes**

Astrocytes play a key role in the regulation of the amount of glutamate in the extracellular environment. Perivascular end-feet, which are astrocytic processes

surrounding microvessels, are believed to take up blood glucose before it reaches the neuron and ultimately convert it into glutamate via one of several enzymes such as glutamate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, or branched-chain aminotransferases<sup>35</sup>. Astrocytes also seem to be the only cell in the brain with the ability to replenish TCA cycle intermediates that are used up in the synthesis of glutamate from alpha-ketoglutarate, a process known as anaplerosis. This process is due to the exclusive presence of pyruvate carboxylase in astrocytes<sup>36</sup>. Astrocytes also significantly influence glutamate catabolism by using the enzyme GS, which is preferentially localized to these cells<sup>37</sup>. While several neuronal vesicular glutamate transporters (VGLUT-1, 2) and excitatory amino acid transporters exist, the majority of synaptic glutamate is taken up by astrocytes and the amino acid is converted to glutamine by GS, then shuttled to neurons, where, once inside, glutamine is converted back to glutamate via phosphate activated glutaminase<sup>38,39</sup>.

### **1.2c GABA**

Gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the brain, and has been implicated in seizure origin and spread<sup>40</sup>. GABA is formed within GABAergic axon terminals by decarboxylation of glutamate. After being released into the synapse GABA can act on one of two types of GABA receptors: GABA-A receptors or GABA-B receptors. GABA-A receptors are ligand-gated ion channels that hyperpolarize neurons by increasing chloride entry into the cell. GABA-A facilitates the early portion of GABA mediated inhibitory post-synaptic potential. GABA-B works by increasing potassium conductance, decreasing calcium entry, and inhibiting the presynaptic release of other transmitters. It is responsible for the late portion of GABA

mediated inhibitory postsynaptic potential. In the Spencer and During microdialysis study cited above, GABA was found to increase during seizures, however, this increase was greater in the non-epileptogenic vs. the epileptogenic hippocampus<sup>29</sup>. They demonstrated the number of GABA transporters is reduced in the epileptogenic hippocampus, and suggested that this reduction impairs non-vesicular GABA release secondary to cell membrane depolarization, resulting in insufficient GABA release to suppress seizure activity<sup>41</sup>. GABA synthesis is impaired in human pyridoxine (vitamin B6) deficiency, a disorder characterized by severe refractory neonatal/infantile seizures<sup>42</sup>. Several animal models have shown the importance of GABA's role in epilepsy as well. For instance, DBA/2 mice, which are particularly susceptible to audiogenic seizures, have reduced numbers of high-affinity GABA receptors, and reduced benzodiazepine binding in multiple brain areas<sup>43,44</sup>. Finally, drugs that enhance GABA concentration in the synapse are often effective ant-epileptic drugs. Vigabatrin, which is an irreversible suicide inhibitor of GABA transaminase, and Tiagabine, which blocks GABA reuptake into neurons and glia, are two such examples.

### **1.2d Inflammation**

Inflammatory processes have also been implicated in epilepsy. Early clinical evidence for inflammation in seizures came from several sources. Febrile seizures, which involve the acute release of cytokines and other inflammatory mediators in the brain, were one early clue<sup>45</sup>. Chronic brain inflammation, such as in Rasmussen's encephalitis, a devastating childhood auto-immune disease, is characterized by severe progressive seizures as well<sup>46</sup>. There is clinical evidence that steroids or other anti-inflammatory treatments can be effective anticonvulsants in some childhood epilepsies that are resistant

to traditional AEDs<sup>47,48</sup>. Finally, several inflammatory mediators are altered in surgically resected hippocampi in patients with TLE<sup>49</sup>.

A question that arises is whether inflammation is a cause or a consequence of epilepsy, and several experimental models have tried to address this. In rats, induction of limbic status epilepticus or recurrent spontaneous seizures results in activation of inflammatory cytokines (IL-1, IL-6, TNF) in the hippocampus, supporting the idea that inflammation might be an intrinsic part of the epileptogenic process<sup>50</sup>. However, it has also been shown that brain inflammation can increase neuronal excitability and predisposition to seizures. There is strong evidence that IL-1 $\beta$ , TNF, IL-6, prostaglandin E2 all play an active role in seizure generation<sup>51</sup>. The complement cascade, leading to formation of the membrane attack complex (MAC), has also been shown to generate seizures when injected sequentially into the hippocampus of rats<sup>52</sup>. These results taken together suggest a cyclical process in which both seizures and inflammation seem to reinforce one another.

### **1.3 Ketogenic Diet**

The ketogenic diet (KD) was originally developed in the 1920s, when relatively few antiepileptic medications were available, to imitate the metabolic effects of starvation after observations that fasting decreased seizure frequency<sup>6</sup>. It had a brief surge in popularity, then was quickly eschewed once the first truly effective AED, phenytoin, was developed in the late 1930s. . With nearly 1/3 of epilepsy patients not adequately controlled on current AEDs, the KD has regained popularity recently as an alternative way to combat medically intractable seizures<sup>53</sup>.

### 1.3a Efficacy of the KD

The initial efficacy studies of the KD were done at Johns Hopkins Hospital in 150 children<sup>54</sup>. Before the study, all children combined had an average number of 410 seizures per month. They had failed to have their seizures controlled despite being previously tried on a mean of 6.2 medications and were on an average of 1.97 medications at the time of diet initiation. After 3 months of treatment with a KD, 34% of the children had >90% decrease in seizures and at 6 months, 32% had a >90% decrease in seizures. One year after beginning the treatment with the KD, 55% of children remained on the diet; 7% were seizure free, and 20% had a 90% decrease in seizures. A randomized control trial of 103 patients was published recently that was similarly efficacious<sup>55</sup>.

The KD has been shown to be effective in a variety of types of epilepsy syndromes and seizure types. The largest clinical trial to date showed no statistically significant difference in efficacy by seizure type<sup>54</sup>. It has been shown to be quite effective, even within the first 48 hours of its application, in decreasing the incidence of atonic or myoclonic seizures in children with Lennox-Gastaut syndrome (LGS)<sup>56</sup>. In a trial of seven girls with Rett syndrome, the KD improved seizure control in 5 of them and also improved social interaction, and reduced stereotypical behaviors<sup>57</sup>. It is felt to be the therapy of choice for Glut-1 deficiency and very effective in Dravet Syndrome and Myoclonic Astatic Epilepsy.<sup>58</sup> A study comparing efficacy of the KD in treating generalized seizures vs. focal seizures showed a tendency towards improved outcome in those with generalized seizures compared to those with focal seizures, but the diet was effective in both types, and no statistical differences were found<sup>59</sup>. Than et al. also



showed that an early, dramatic response to the KD (defined as those patients becoming seizure free after two weeks on the KD) was most often observed in generalized seizures, rather partial seizures in children.<sup>60</sup>

Though traditionally thought of as a treatment for pediatric epilepsies, there is evidence that the KD works in adults as well. Kossoff et al tried a group of 30 adults who had at least weekly seizures and had failed at least 2 AEDs on a modified Atkins diet (a less restrictive KD) and obtained encouraging results<sup>61</sup>. At 1 and 3 months, 47% of patients had a >50% seizure reduction, and after 6 months on the diet, 33% had a >50% seizure reduction. Previously, it was believed that the KD is more effective in children due to an enhanced ability to extract ketones from the blood or due to their metabolic capacity but may be due to poor tolerance of the diet in adults.

Importantly, the diet has also been shown to have antiepileptic effects (i.e. interfering with the process of developing recurrent unprovoked seizures) in addition to the previously discussed anticonvulsive effects. In a 3 to 6 year follow up of children who had been on the KD, Marsh et al. showed that children who discontinued the diet, some after only a few months, had long-term reductions in seizure even after the diet was stopped.<sup>62</sup>

### **1.3b Practical Issues and Adverse Effects of the KD**

One obstacle in the use of the KD in adults is compliance with the strict dietary guidelines. In a small study in which the full KD was tried on adults, 4 of the 11 had discontinued the diet by 8 months follow-up<sup>63</sup>. The most challenging aspect of the KD for most people is abstaining from carbohydrates. The traditional KD is a 4g fat/1g

combined protein and carbohydrate<sup>64</sup>. Most centers, when initiating the diet, hospitalize patients while they undergo an initial phase of fasting to achieve ketosis<sup>65</sup>. Once robust ketosis is confirmed, patients begin the KD and are supplemented with a fat-soluble vitamin supplement and additional calcium. Table 1 shows a sample KD for 1 day.

Table 1. Sample ketogenic diet for 1 day (from Sirven et. al)

<b>Breakfast</b>	<b>Lunch</b>	<b>Dinner</b>
2 medium eggs	2 hot dogs	Chicken 1.5 ounces
1 sausage patty	2 lettuce leaves, 1 thin slice tomato	½ cup green beans, 2 tsp butter
11 tsp butter	3.5 tbs mayonnaise	4 tbl vegetable oil
3 tbs heavy whipping cream		

Side effects of the KD are rarely so severe that the diet must be discontinued.<sup>66</sup> Acidosis, hypoglycemia, gastrointestinal distress, dehydration, and lethargy can occur early after treatment with the KD is begun. These effects are typically short lived when they do occur and can be relatively easily managed.

Longer-term adverse effects include increases in blood lipid levels and cholesterol. After 6 months on the diet LDL levels increased an average of 50 mg/dL, or 2 standard deviations in a group of 141 children<sup>67</sup>. Triglycerides and atherogenic apo-B containing lipoproteins increased and HDL levels decreased in this same group. However Kossoff has shown that lipid levels normalize with time and there appears to be no evidence of increased cardiovascular or hepatic disease.<sup>68</sup>

Kidney stones are another longer-term complication of the KD. They occur in

about 6% of patients and present, on average, after about 7 months on the KD.<sup>69</sup>

Children on the KD are at increased risk for kidney stones for a number of reasons. The diet creates an acidosis which promotes hypercalciuria, secondary to increased bone demineralization, hypocitrauria, and low urine pH.<sup>70</sup> In addition, many patients on the diet are fluid restricted, which can lead to supersaturation of urine with calcium or uric acid, creating a nidus for stone formation. The risk of kidney stones can be decreased with supplementation with potassium citrate

Children on the diet gain minimal weight and height within the normative values for their age<sup>71</sup>. However, the growth of young children (<1 year) seems to be slowed more than that of older children, and they should be monitored carefully. Most children on the KD for more than 6 years were in the bottom 10% for height and weight.<sup>72</sup> Though there is some data to suggest that children grow rapidly once the diet is discontinued.<sup>73</sup>

### **1.3c Possible Mechanism of Action of the KD**

As mentioned above, the classic ketogenic diet consists of a 4:1 ratio of fats to protein and carbohydrates. Severely restricting carbohydrates from the diet leads to glycogen depletion in the liver and decreased glucose utilization by the body. In lieu of glucose, fatty acids are metabolized for energy. Beta-oxidation of fatty acids takes place in mitochondria in the liver, the end product of which is acetyl-CoA. When TCA cycle intermediates are depleted, as they are in long-term carbohydrate restriction, acetyl-CoA accumulates and is shuttled down the ketogenesis pathway, producing the ketone bodies: acetoacetate, acetone, and  $\beta$ -hydroxybutyrate. These ketone bodies become the major fuel source for the brain while on the KD.

Though a number of different mechanisms of action of seizure reduction have been proposed for the diet, there is no consensus currently which hypothesis is correct.<sup>74</sup> The ketone bodies themselves have been a major area of investigation. Peterman, in his preliminary report of KD, postulated that the acetoacetate acid acts as an anesthetic agent that penetrates the CNS to suppress seizures.<sup>64</sup> More modern experience with the diet has shown that the anticonvulsant effect rises slowly, as blood levels of ketones rise, and then fall off abruptly if ketosis is eliminated by ingesting carbohydrates.<sup>75</sup> Additionally,  $\beta$ -hydroxybutyrate blood levels appears to correlate with seizure control in some individuals.<sup>76</sup> And in animal models, direct injection of ketone bodies have been shown to be anticonvulsant in mice with audiogenic seizures.<sup>77</sup> These findings, together, seem to hint at a direct role for ketone bodies in seizure reduction.

### **Inhibition of vesicular glutamate loading**

If ketone bodies are directly limiting seizure activity, what are they targeting? Recently research has shown that ketone bodies are physiological modulators of VGLUT, which transports glutamate into synaptic vesicles. When cultured neurons were perfused with acetoacetate at a concentration of 1 mM (a concentration expected in humans during the KD) VGLUT2 was inhibited.<sup>78</sup> Acetoacetate competes for the  $\text{Cl}^-$  binding site on VGLUT, turns the transporter off upon binding, leading to decreased glutamate storage in vesicles, and therefore less glutamate release by neurons. In hippocampal slices, acetoacetate was shown to reduce excitatory glutamatergic synapse transmission in CA1 neurons. Additionally, when the proconvulsant  $\text{K}^+$  channel blocker 4-aminopyridine was infused in rats, neuronal hyperexcitability was reversed by direct infusion of acetoacetate.<sup>78</sup>

### **Reducing neural excitability via $K_{ATP}$ channels**

One consequence of ketone body metabolism in neurons is increased ATP production by mitochondria and a decrease in glycolytic ATP production.<sup>79</sup> Earlier work has shown that glycolytic ATP is synthesized by membrane bound enzymes and deposited in membrane-associated compartments, where it can be used by  $Na^+/K^+$  pump.<sup>80</sup> When ketone body metabolism shifts the bulk of ATP production to the mitochondria, ATP reserves in membrane-associated compartments are depleted. This decrease in membrane associated ATP leads to opening of the membrane bound  $K_{ATP}$ , which in turn leads to hyperpolarization and decreased excitability.<sup>81</sup> Yellen et al have shown that when acetoacetate or  $\beta$ -hydroxybutyrate are applied to brain slices in a constant level of glucose, spontaneous firing is decreased by approximately 15%.<sup>82</sup> Spontaneous firing was decreased further on cells that fired faster, known as “use dependence”. They also demonstrated that the decreased excitability effect is eliminated in  $K_{ATP}$  blocking drugs are used or when the Kir6.2 subunit is knocked out genetically.

The role of  $K_{ATP}$  channels in the seizure suppressing effects of the KD have been further explored using BAD knock out mice.<sup>83</sup> BCL-2- associated Agonist of Cell Death (BAD) is a pro-apoptotic member of the BCL-2 family that regulates the release of apoptogenic factors from mitochondria. In addition to its role in apoptosis, it has recently been shown that BAD modulates glucose metabolism via phosphorylation of the serine 155 residue on glucokinase in multiple types of cells.<sup>84</sup> When BAD is knocked out in mice, glucose metabolism is reduced and cells undergo changes in metabolism similar to that seen during ketone body metabolism.<sup>83</sup> Furthermore, mice with BAD knocked out have been shown to be resistant to seizures caused by kainic acid and by

pentylentetrazole (PTZ), a GABAergic antagonist. The open probability for  $K_{ATP}$  channels was significantly increased in these mice. Additionally, when  $K_{ATP}$  channel was made dysfunctional by deletion of the Kir6.2 subunit, the BAD knockout mice reverted back to wild type seizure sensitivity, offering compelling evidence for  $K_{ATP}$  mediating BAD's effect on neuronal excitability.<sup>83</sup>

## **MCT1**

Upregulation of the monocarboxylate transporter 1 (MCT1) is another possible mechanism of action of the KD. MCT1 is found throughout the brain and is a key transporter of blood derived monocarboxylates such as pyruvate, lactate and the ketone bodies acetoacetate and beta-hydroxybutyrate.<sup>85</sup> MCT1 is also a transporter of acidic drugs, such as the antiseizure medication valproic acid, across the blood brain barrier.<sup>86</sup> Eid and colleagues recently showed that patients with TLE were severely deficient in MCT1 on the endothelial cell membrane of microvessels in the epileptogenic hippocampal formation.<sup>87</sup> This finding suggests that the uptake of blood-derived monocarboxylate fuels and possibly also antiepileptic drugs are impaired in the seizure focus of the brain. The loss of MCT1 on microvessels has been reproduced in three novel rat models of TLE, indicating that MCT1 is of key importance in the pathophysiology of the disease.<sup>88</sup>

Interestingly, Leino in 2001 showed that adult, non-epileptic rats, when fed a KD, upregulate brain MCT1 by 8 fold.<sup>85</sup> A key question therefore is whether the therapeutic action of the KD in epilepsy can be explained by an upregulation of MCT1 in epileptogenic areas of the brain. It is yet to be shown, however, that MCT1 levels are

increased in response to a KD in epilepsy.

#### **1.4 MSO Model of TLE**

Over the years there have been many methods of provoking seizures in animals. However, it is critical that disease oriented translational science experiments reflect the underlying human pathology as closely as possible, and as consistently as possible. Systemic administration of kainic acid or injection of pilocarpine results in severe status epilepticus, however the seizures can be lethal and widespread brain damage, which is not a feature of TLE, is often seen as well.<sup>89,90</sup> Models such as amygdala kindling and prolonged hyperthermia produce seizures, but often do not result in spontaneous, recurrent seizures.<sup>91,92</sup> However, the MSO model, in which MSO is continuously infused unilaterally into the hippocampus, replicates many of the key features of TLE. Episodes of spontaneous, recurrent seizures – many of these are partial – with a low mortality rate are consistently seen in this model. Pathological examination has shown a range of histologies, from little to no neuronal loss in the hippocampus to hippocampal sclerosis-like pathology, to extensive neural degeneration and gliosis. MSO acts by inhibiting glutamine synthetase, which has been shown to be deficient in resected hippocampi of humans with TLE, so its mechanism of action may be relevant to one possible etiology of TLE as well.

## **Chapter 2: Hypothesis**

We hypothesize that the ketogenic diet combined with 20% caloric restriction will reduce seizure burden in the rat MSO model of TLE.

### ***Specific Aims:***

- 1.) To assess the effect of the KD and caloric restriction on the total number of seizures in the rat MSO model of TLE
- 2.) To assess the effect of the KD and caloric restriction on the severity of seizures in the rat MSO model of TLE



## **Chapter 3: Methods**

### **3.1 Chemicals and Animals**

All chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted. Adult, male Sprague Dawley rats were used in this study (200 to 250 g; Charles River Laboratories, Wilmington, Mass.). The rats had free access to water and were housed on a 12-h light/dark cycle, with lights on from 7 a.m. to 7 p.m. The animal care and use procedures were approved by the Institutional Animal Care and Use Committee of Yale University. All experiments were performed in accordance with current guidelines.

### **3.2 Diet**

After implantation of the MSO cannula, rats were divided into two groups: an experimental group fed a ketogenic diet with 20% caloric restriction (Ketovolve, Solace Nutrition, CT, n=12), and a control group fed standard rodent chow (SC) (Teklad Rodent Diet, Harlan, n=26) ad libitum. The experimental group was given a ketogenic diet consisting of 89% fat and 9% protein (based on caloric content) and 20% caloric restriction for 3 weeks in one set of animals, and 4 weeks in another. The diet was started one week after cannula implantation and MSO infusion and continued until the rats were euthanized. This diet has been shown to produce significant ketosis in previous studies.<sup>85,93</sup>

Normal caloric intake was determined by monitoring the rats' caloric intake for 1 week prior to surgery. At the end of 1 week the average daily caloric intake was calculated, then reduced by 20%. For example, 300g of standard chow was placed in the

rat's feeder. Seven days later the food was removed and 121g remained. The rat ate 178g, which averages to 25.5g/day or 84kcal/day. Reducing the calories by 20%, this rat would be fed 67kcal/day, or 9.5g/day of Ketovolve powder. After an initial fasting period of 24 hours, the rats on the KD were fed once per day. See table 2 for comparison of the caloric contents of the diets.

Table 2. Comparison of macronutrients of standard chow vs. KD

Diet	kcal/g	% Protein	% Fat	% Carbs
Standard Chow	3.3	20	13	67
Ketogenic Diet	7.1	8.7	89.2	2.1

### 3.3 Surgery

The rats were anesthetized with 1% to 2% Isoflurane (Baxter, Deerfield, Ill.) in O<sub>2</sub> and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, Calif.). A 30-gauge stainless steel cannula attached to a plastic pedestal (Plastics One, Roanoke, Va.) was introduced through a burr hole in the skull and into the right hippocampus, using the following coordinates with bregma as the reference: AP=-5.6 mm, ML=5.3 mm, DV=-6.5 mm. The cannula was cemented to the skull using cyanoacrylate and connected via plastic tubing to a subcutaneously implanted Alzet osmotic pump (Model 2004, Durect Corp., Cupertino, Calif.). This pump holds a total volume of 200  $\mu$ L and delivers a continuous flow of 0.25  $\mu$ L/h for ~28 days (as per manufacturer's specifications). The pumps were filled with MSO (2.5 mg/mL; dissolved in 0.9% NaCl) to achieve a delivery

of 0.625  $\mu\text{g}$  of MSO per hour. MSO delivery was started as soon as surgery was completed.

Two unipolar electrodes (E363/2/SPC stainless steel electrode, Plastics One) with bare diameter of 0.200 mm and insulated diameter of 0.230 mm, were introduced into the dorsal hippocampus to record continuous bilateral intrahippocampal EEG activity in freely moving, awake animals. Approximately 1 mm of insulation was stripped from the tip of each electrode. The coordinates used were as follows: AP = - 3.3 mm, ML = 2.5 mm, DV = - 3.9 mm. One electrode was inserted into each hippocampus. A third depth electrode was positioned in the epidural space near lambda to serve as the reference. A screw electrode was positioned in the occipital bone to serve as the ground.

The female socket contacts on the end of each electrode were inserted into a plastic pedestal (Plastics One), and the entire implantation was secured by UV light cured acrylated urethane adhesive (Loctite 3106 Light Cure Adhesive, Henkel Corp., Rocky Hill, Conn.).

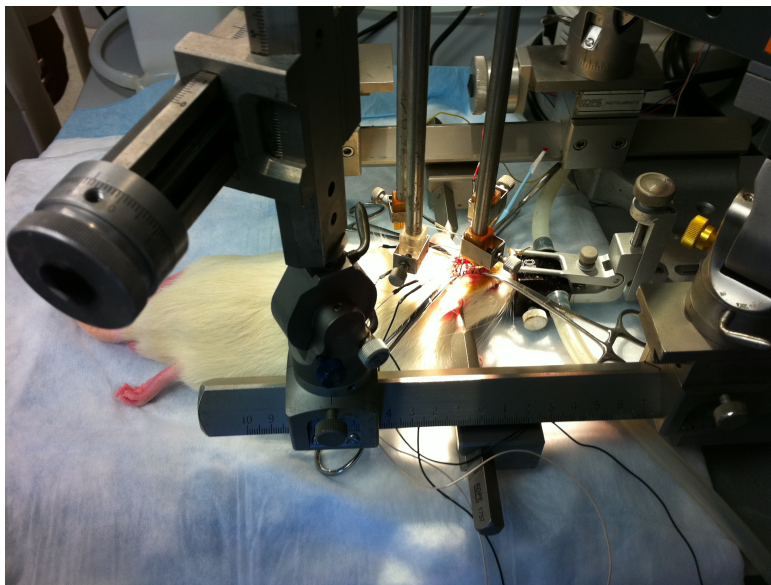


Image 1. Rat in stereotactic frame undergoing surgery.

### **3.4 Different length of recording times**

We initially recorded all of the animals for 3 weeks post surgery, i.e. 1 week on standard chow and 2 weeks on the KD. After analyzing the data, we saw no significant difference in the number or severity of seizures between the groups. However, there was a trend towards decreased severity of seizures during the 3<sup>rd</sup> week. We decided extended the study to 5 weeks (i.e. 1 week on standard chow, 4 weeks on the KD) in subsequent animals, in order to investigate the trend over a longer time period. As a result, there are fewer animals in the study during the 4<sup>th</sup> and 5<sup>th</sup> week.

### **3.5 Video-intracranial EEG monitoring**

The experimental setup for recording video-EEG was adapted from Bertram et al.<sup>94</sup> The rats were placed individually in custom-made Plexiglas cages. A spring-covered, 6-channel cable was connected to the electrode pedestal on one end and to a commutator (Plastics One) on the other. A second cable connected the commutator to the digital EEG recording unit (Ceegraph Vision LTM, Natus Bio-logic Systems Corp., Mundelein, Ill.). Digital cameras with infrared light detection capacity were used to record animal behavior (two cages per camera). The digital video signal was encoded and synchronized to the digital EEG signals. Seizures were identified by visual inspection of the EEG record. Seizures were defined by EEG characteristics and not by the duration of the discharge. Specifically, seizures displayed distinct signal changes from background (interictal) activity. Such signal changes included sustained rhythmic or spiking EEG patterns and a clear evolution of signal characteristics from onset to termination.

Subclinical seizures were distinguished from clinical seizures by examination of the video record. The start and stop points of seizures were identified by the following commonly used method. By visual inspection of the EEG, we determined a point that was unequivocally within the seizure. Next we moved backward in time to determine the seizure start time as the first point where the EEG was different from background activity and forward in time to establish the seizure end time. The video record was examined to stage the seizures, using a modification of Racine's criteria, as follows: Subclinical, no remarkable behavior; stage I, immobilization, eye blinking, twitching of vibrissae and mouth movements; stage II, head nodding, often accompanied by facial clonus; stage III, forelimb clonus; stage IV, rearing; stage V, rearing, falling and generalized convulsions.<sup>95</sup>



Image 2. Post-surgery rat in Plexiglas cage undergoing continuous video and intracranial EEG monitoring. The rat is exhibiting a stage IV seizure with rearing.

### **3.6 Blood glucose and ketone monitoring**

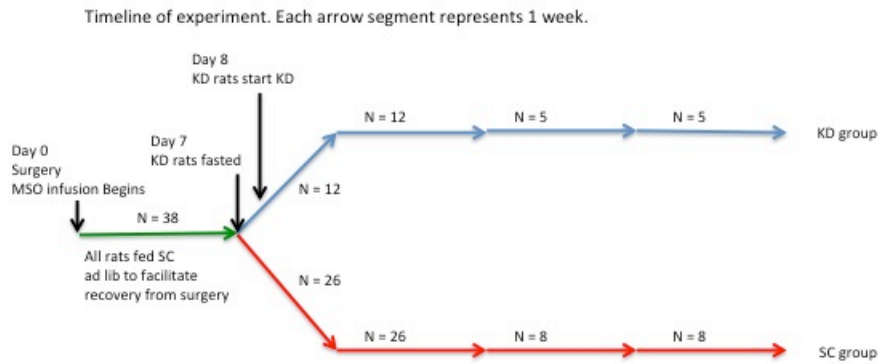
Ketone and glucose levels were taken from rats in the afternoon prior to being fed on a weekly basis starting the day of surgery (day 0) and continued until perfusion date. Rats were anesthetized using 1-2% isoflurane prior to being pricked by an 18g needle at the end of its tail to draw blood. Between rats, the needle was cleaned with alcohol. The first drop of blood drawn was wiped off and discarded. Blood ketone levels were measured using a Precision Xtra Blood Glucose and Ketone Monitoring System (Abbott, Alameda, CA) with blood  $\beta$ -Ketone test strips. Blood glucose levels were measured using an AlphaTRAK Blood Glucose Monitoring System (Abbott Laboratories North Chicago, IL) with glucose test strips for rats and mice. Each rat's blood glucose and ketones were measured and recorded with the time of day before the next rat was anesthetized and the process was repeated for the next rat.

### **3.7 Statistical Analysis**

A two group comparison using students two way student's t-test was used to analyze the data with statistical significance determined using the Holm-Sidak method, with alpha = 5%. This was carried out in GraphPad Prism version 6.0 for mac OS X, GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com).

### 3.8 Experimental Timeline

Fig 1. Timeline of experiment



## Chapter 4: Results

### 4.1 Verification and Temporal Distribution of Seizures

We first established that all MSO-infused rats developed recurrent seizures, i.e.  $\geq 2$  seizures each separated by at least 1 h. An initial group of 18 standard chow (SC) control and 7 KD rats were studied for 21 days. A second group of 8 SC control and 5 KD rats were studied for 35 days. All rats (38 of 38) were monitored by continuous video and hippocampal depth electrode EEG recordings, and all of the rats developed recurrent seizures.

We then assessed the temporal distribution of the seizures. Thirty-three (87%) of the animals exhibited an initial cluster of recurrent seizures that began within the first 48 h after the initiation of MSO infusion (Fig. 1). The highest mean frequency of seizures during the initial cluster was observed during days 1 (12.9 seizures per 24h, range = 0 to 73) and 2 (9.8 seizures per 24h, range, 0 to 71 ; Fig. 2). Then during day 3 the seizure frequency began to decline markedly, from an average of 3.4 seizures per 24 h (range, 0 to 50) to an average of 1 seizure per 24 h (range, 0 to 12) on day 4. After the initial cluster of seizures, on days 6 and 7 there was an increase in the mean number of seizure to 2.9 and 3.7 respectively, before declining again in an asymptotic manner towards zero. This distribution of seizures is similar to what has been seen before in previous studies using the MSO model.<sup>96</sup>





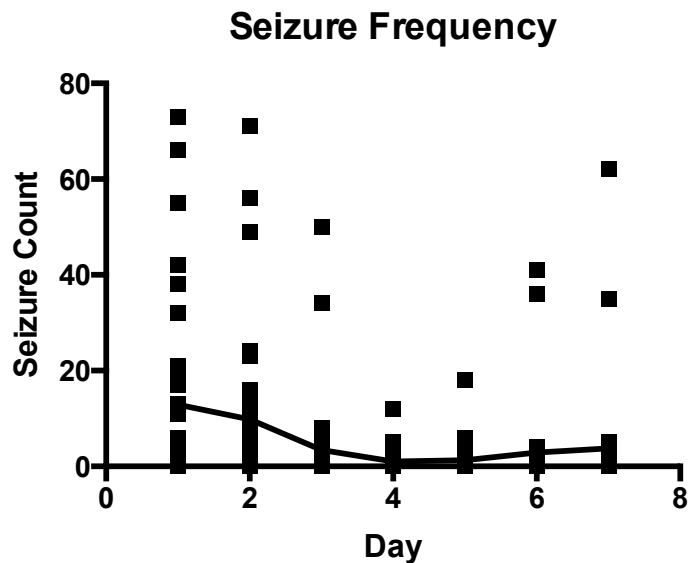


Fig 3. Plot of the seizure frequency for all animals for the first seven days after MSO infusion. The line drawn through the boxes represents the average seizure frequency for that particular day. Note the cluster of initial seizures during days 1-2, followed by a decline in seizure frequency during days 3-5, with subsequent smaller increase during days 6-7.

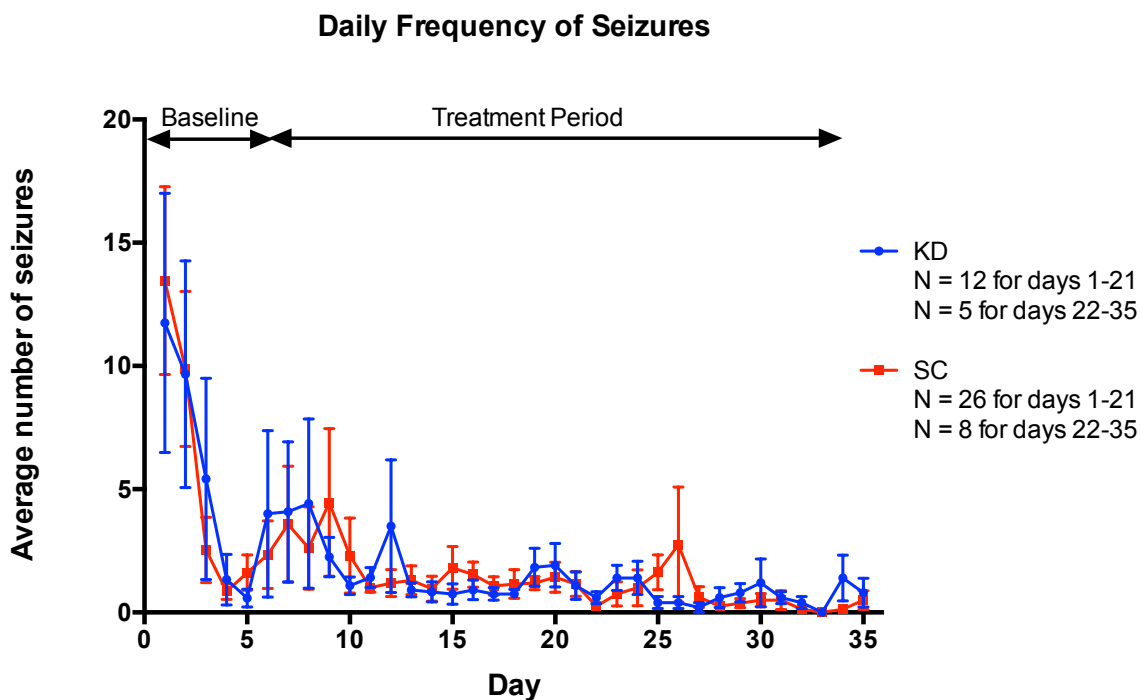


Fig 4. Average number of seizures each day for both groups of animals. The greatest number of seizures for both groups occurred during the first 3 days of the study; there is a nadir around day 5, then another peak days 6-9, followed by a slow taper

## 4.2 Effect of KD on Seizure Frequency

Next we evaluated the effect of the KD on the frequency of seizures in the MSO-infused rats. This was done by binning the total number of seizures in intervals of 1 week followed by statistical analysis using a student's t-test (Fig. 4a). Surgery was done on day 0, and both groups (SC and KD) were fed a standard diet ad libitum during week 1 to facilitate recovery from surgery. A KD with 20% caloric restriction was initiated in the treatment group on week 2. An initial study was carried out for 3 weeks (with 7 KD and 18 SC rats), and then a second study was carried out for 5 weeks (with 5 KD and 8 SC rats), accounting for the smaller N in the graphs during weeks 4 and 5.

We found no significant difference in the weekly frequency of seizures in rats on the KD versus rats on SC (Table 3). During the first week, both groups had similar mean numbers of seizures (SC:  $34.3 \pm 7.6$ , KD:  $36.8 \pm 13.8$ ), which would be expected because both groups were fed normal chow ad libitum. Both groups continued to exhibit similar mean numbers of seizures during week 2 (SC:  $13.8 \pm 6.3$ , KD:  $14.4 \pm 4.6$ ). During weeks 3 and 4, the KD group had 14% (1.3), and 32% (2.3) fewer mean number of seizures than the SC group, though the difference was not statistically significant ( $p=0.70$ , and  $p=0.61$ , respectively). During week 5, the KD group paradoxically had more seizures (SC:  $2.1 \pm 0.8$ , KD:  $5.2 \pm 1.8$ ), but again, this was not statistically significant ( $p=0.11$ ).

	KD: mean # of all seizures $\pm$ SEM	SC: mean # of all seizures $\pm$ SEM	p-value
Week 1	36.8 $\pm$ 13.8	34.3 $\pm$ 7.6	0.86
Week 2	14.4 $\pm$ 4.6	13.8 $\pm$ 6.3	0.95
Week 3	8.0 $\pm$ 1.9	9.3 $\pm$ 2.1	0.70
Week 4	5.0 $\pm$ 1.2	7.3 $\pm$ 3.2	0.61
Week 5	5.2 $\pm$ 1.8	2.1 $\pm$ 0.8	0.11

Table 3. Comparison of the average number of seizures for KD vs. standard chow (SC) treated rats. The number of seizures were binned in one week intervals. During week 1, all rats are fed SC ad libitum. Treatment with the KD began during week 2. Student's t-test showed no significant reduction of seizures in any week with statistical significance determined using the Holm-Sidak method, with  $\alpha = 5\%$ .

### 4.3 Effect of KD on Seizure Severity

Next we evaluated the effect of the KD on the behavioral severity of seizures.

This was done by examining the video record to stage the seizures, using a modification of Racine's criteria, as described in the *methods*. The weekly frequency of seizures at each of the behavioral stages was compared between the KD and SC treated rats using a student's t-test.

We found no significant difference in the severity of seizures between rats treated with the KD and rats fed SC (Fig. 4b-f. and Table 4) using student's t-test with statistical significance determined using the Holm-Sidak method, with  $\alpha = 5\%$ .

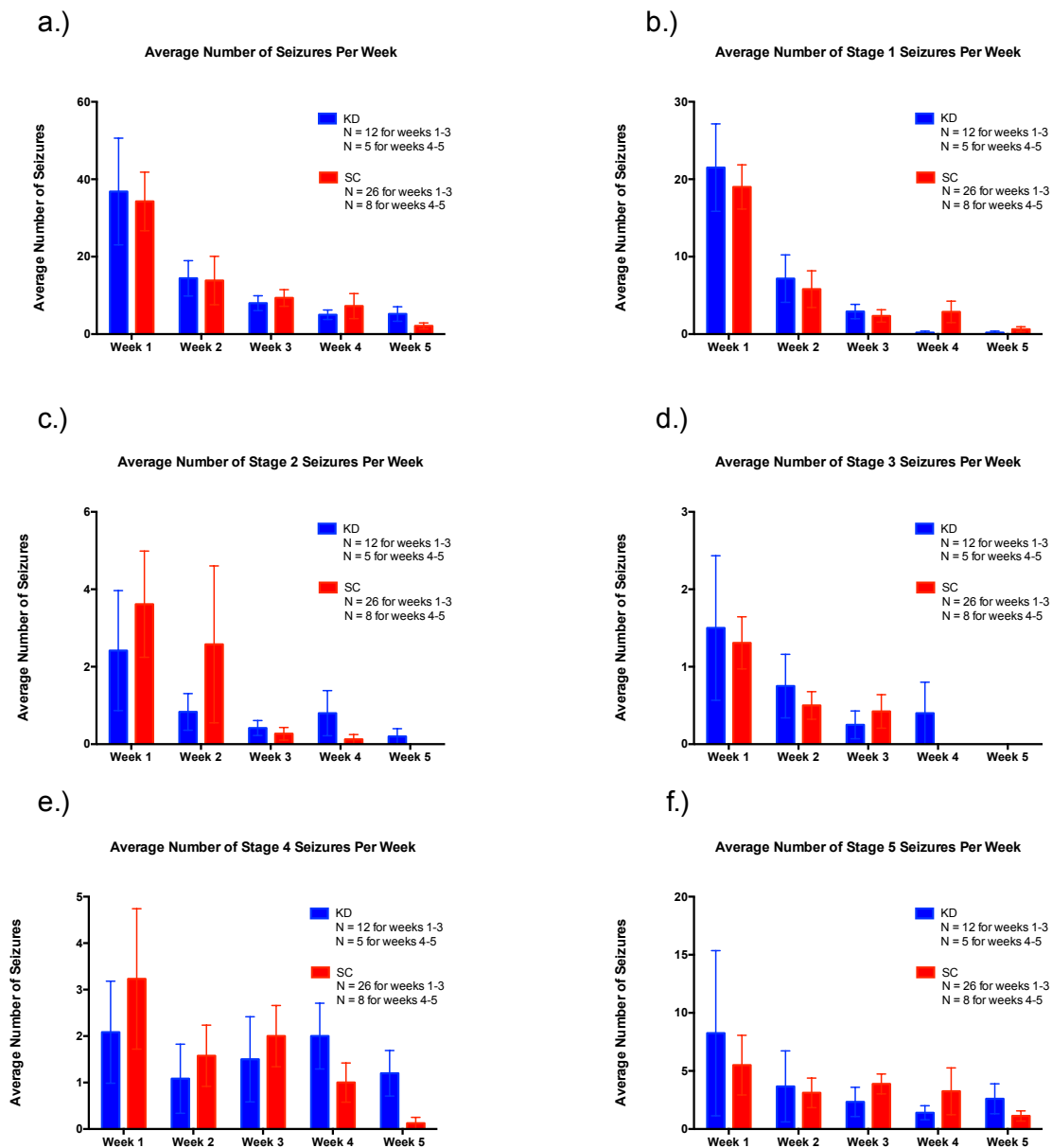


Fig. 5. Weekly frequency of all seizure types (a) and of seizure types at different behavioral severities (b-f) in KD treated (blue bars) and standard chow (SC, red bars) rats. All rats were given SC during week 1. KD started on week 2. Shown is the mean number of seizures. There were no significant differences in seizure frequency or severity between KD versus SC treated rats.

#### 4.4 Table of Results for Seizure Severity

For table 4 below, the number of seizures were sorted by severity and binned in one week intervals. During week 1 all rats are fed standard chow ad libitum. Treatment with the KD began during week 2. Shown below is the mean  $\pm$  SEM number of seizures for each week and the corresponding p-value. Student's t-test showed no significant reduction of seizures in any week with statistical significance determined using the Holm-Sidak method, with alpha = 5%. The p-value for stage 4 seizures during week 5 was  $p=0.02$ , however using the Holm-Sidak method of correcting for multiple comparisons, the values was not significant.

	KD: mean # of seizures $\pm$ SEM	SC: mean # of seizures $\pm$ SEM	p-value
	<b>Stage 1</b>	<b>Stage 1</b>	
Week 1	21.5 $\pm$ 5.6	19.0 $\pm$ 2.9	0.66
Week 2	7.2 $\pm$ 3.1	5.8 $\pm$ 2.4	0.74
Week 3	2.9 $\pm$ 0.9	2.3 $\pm$ 0.8	0.67
Week 4	0.2 $\pm$ 0.2	2.8 $\pm$ 1.4	0.16
Week 5	0.2 $\pm$ 0.2	0.6 $\pm$ 0.3	0.36
	<b>Stage 2</b>	<b>Stage 2</b>	
Week 1	2.4 $\pm$ 1.5	3.6 $\pm$ 1.3	0.60
Week 2	0.8 $\pm$ 0.5	2.6 $\pm$ 2.0	0.57
Week 3	0.4 $\pm$ 0.2	0.3 $\pm$ 0.2	0.59
Week 4	0.8 $\pm$ 0.6	0.1 $\pm$ 0.1	0.18
Week 5	0.2 $\pm$ 0.2	0.0 $\pm$ 0.0	0.22
	<b>Stage 3</b>	<b>Stage 3</b>	
Week 1	1.5 $\pm$ 0.9	1.3 $\pm$ 0.3	0.81
Week 2	0.8 $\pm$ 0.4	0.5 $\pm$ 0.2	0.52
Week 3	0.3 $\pm$ 0.2	0.4 $\pm$ 0.2	0.62
Week 4	0.4 $\pm$ 0.4	0.0 $\pm$ 0.0	0.23
Week 5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	n/a
	<b>Stage 4</b>	<b>Stage 4</b>	
Week 1	2.1 $\pm$ 1.1	3.2 $\pm$ 1.5	0.63
Week 2	1.1 $\pm$ 0.7	1.6 $\pm$ 0.7	0.66
Week 3	1.5 $\pm$ 0.9	2.0 $\pm$ 0.7	0.67
Week 4	2.0 $\pm$ 0.7	1.0 $\pm$ 0.4	0.21
Week 5	1.2 $\pm$ 0.5	0.1 $\pm$ 0.4	0.02
	<b>Stage 5</b>	<b>Stage 5</b>	
Week 1	8.2 $\pm$ 7.1	5.5 $\pm$ 2.6	0.65
Week 2	3.7 $\pm$ 3.1	3.1 $\pm$ 1.3	0.84
Week 3	2.3 $\pm$ 1.3	3.8 $\pm$ 0.9	0.32
Week 4	1.4 $\pm$ 0.6	3.3 $\pm$ 2.0	0.50
Week 5	2.6 $\pm$ 1.3	1.1 $\pm$ 0.4	0.22

Table 4. Mean number of seizure for KD and SC rats sorted by severity.

#### 4.5 Blood Glucose and Ketone Levels

Blood glucose and ketone levels were measured throughout the experiment, starting on the day of surgery (day 0) in a random selection of rats. As can be seen from the graphs in Figure 5, prior to the start of the diet, both groups of rats had similar blood glucose and ketone levels (give values). The KD was started on day 8, and significant differences in blood glucose levels between the two groups were seen in measurements taken after the start of the diet (days 14, 21, 28). Significant differences in blood ketone levels are seen between the two groups in days 21 and 28. There were no measurements taken of ketone levels in the diet group on day 14. No blood glucose or ketone measurements were taken in standard chow rats on day 35 (Table 5)

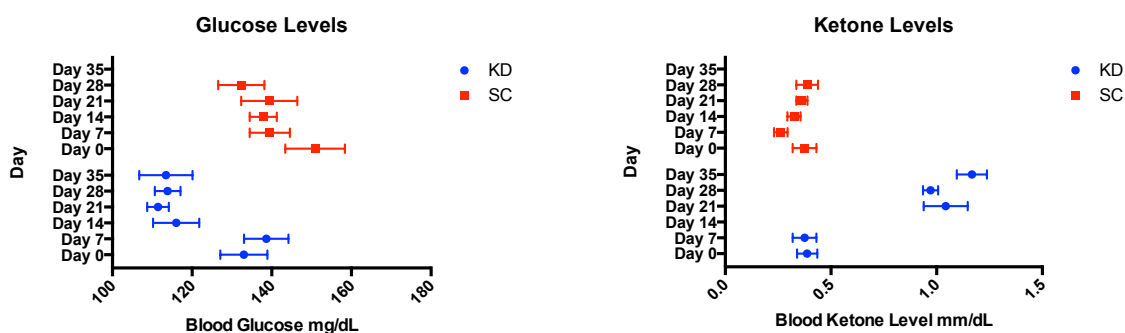


Figure 6. Blood glucose and ketone levels for a random selection of 8 rats in the KD and SC treated groups. Both groups were fed standard chow ad libitum for 7 days to facilitate recovery from surgery. The diet group started treatment on day 8. The first measurement taken while the rats were on the KD was on day 14. KD rats had significantly decreased blood glucose levels on days 14, 21, and 28. KD rats had significantly increased blood ketone levels on days 21 and 28.



	KD: Mean Blood Glucose Levels mg/dL $\pm$ SEM	SC: Mean Blood Glucose Levels mg/dL $\pm$ SEM	p-value
Day 0	133 $\pm$ 5.9	151 $\pm$ 7.5	0.08
Day 7	138 $\pm$ 5.6	140 $\pm$ 5.0	0.91
Day 14	116 $\pm$ 5.8	137 $\pm$ 3.4	0.005*
Day 21	111 $\pm$ 2.7	139 $\pm$ 7.0	0.004*
Day 28	114 $\pm$ 3.2	132 $\pm$ 5.8	0.019*
Day 35	113 $\pm$ 6.7	No Data	N/A

Table 5. Mean blood glucose levels for KD and SC rats. Significant differences are marked with an asterisk.

	KD: Mean Blood Ketone Levels mm/dL $\pm$ SEM	SC: Mean Blood Ketone Levels mm/dL $\pm$ SEM	p-value
Day 0	0.39 $\pm$ 0.05	0.38 $\pm$ 0.06	0.87
Day 7	0.38 $\pm$ 0.06	0.26 $\pm$ 0.03	0.10
Day 14	No Data	0.33 $\pm$ 0.03	N/A
Day 21	1.04 $\pm$ 0.10	0.36 $\pm$ 0.03	< 0.0001*
Day 28	0.97 $\pm$ 0.04	0.39 $\pm$ 0.05	<0.0001*
Day 35	1.17 $\pm$ 0.07	No Data	N/A

Table 6. Mean blood ketone levels for KD and SC rats. Significant differences are marked with an asterisk.

## Chapter 5: Discussion

TLE is a particular devastating form of epilepsy. It is often drug resistant despite treatment with appropriate anti-epileptic drugs. Due to the unpredictable timing of seizures in this disease, patient with uncontrolled epilepsy experience a decreased quality of life with significant psychological, economical, and social impact. Thus, there is a need for greater understanding of the pathophysiology of TLE and a necessity to develop more efficacious therapies for this disease.

The KD was developed in the 1920s, after observation that fasting decreased seizures in some patients, and before the first truly effective AED, phenytoin, was discovered. Since the discovery of phenytoin there have been many new AEDs come on the market, yet up to 25% of all adults with epilepsy, and 40% of patients with TLE have uncontrolled seizures, despite treatment with multiple drugs<sup>97</sup>. Lack of effective drugs has led to a renewed interest in the KD as a treatment for refractory seizures. The KD is a high-fat, low carbohydrate diet that has been shown in randomized controlled trials to be effective in reducing seizure burden in patients with refractory epilepsy<sup>55</sup>.

In this study we examined the effects of the KD on a highly relevant rat model of TLE. Continuous infusion of MSO, an inhibitor of glutamine synthetase, unilaterally into the hippocampus in rats consistently results in episodes of recurrent, mainly partial seizures, as shown here and in earlier studies<sup>96</sup>. This model also produces neuropathological changes seen in human TLE, such as hippocampal sclerosis and selective loss of MCT1 on microvessels in the hippocampus.<sup>87</sup>

The general trend of seizures for both the SC and KD treated rats showed the

greatest number of seizures occurring during the first 3 days of the study; there is a nadir around day 5, then another peak days 6-9, followed by a slow taper. There is a trend towards the development of more severe seizures as time goes on. That is, the majority of seizures during the first week are stage 1 events. By week 5, however, the majority of seizures are stage 4 and 5. We found no significant difference in the number or severity of seizures between the two groups in this study.

Why was the KD ineffective in this trial? Despite relatively strong evidence of the effectiveness of the KD in humans, evidence of the effectiveness of the KD in animal models is much less robust – results have been model dependent, age dependent, and occasionally contradictory. The first animal study to look at prolonged treatment with the KD was done by Uhlemann and Neims in 1972 on mice, using a number of different techniques to induce seizures.<sup>98</sup> They found that a 3:1 KD (70% fat) protected mice from bicuculline, maximum electroshock and hydration electroshock seizures, but not against threshold electroshock or pentylenetetrazole induced seizures. They also found that the diet had an anticonvulsive effect only in neonatal mice. This is similar to the results of other studies that have found an age dependent effect of the KD in animal models.<sup>99</sup>

Mahoney et al found that magnesium deficient, audiogenic-induced seizure rats treated with the KD surprisingly had increased seizure severity and decreased seizure latency.<sup>100</sup> In another paradoxical result, 20 day old rat pups weaned on a medium chain triglyceride diet for 10 day before they were subjected to maximum electroshock, threshold electroconvulsive shock, threshold pentylenetetrazole, or maximum pentylenetetrazole, no protective effect of the KD was observed; and the KD was actually proconvulsant in tests involving maximal seizure, despite blood levels of BHB that were

comparable or higher to those commonly reported in clinical studies.<sup>101</sup>

In a similar study comparing the effectiveness of the KD in threshold seizure tests (pentylentetrazol infusion test and the electroconvulsive shock test threshold test) to suprathreshold seizure tests (maximal pentylentetrazol test and maximal electroshock test) and found KD was ineffective in reducing seizures in suprathreshold test, but did reduce seizures, albeit slightly, in threshold test.<sup>102</sup> The authors posit that the KD produces a small elevation in seizure threshold and only works when the stimulus does not greatly exceed the seizure threshold. The end result of these studies is that we still do not have a clear picture when or how the diet works in animal models, but results seem to be sporadic and dependent upon a number of factors. Bough and Rho, reviewing the literature of animal studies using the KD, came away with the following generalizations: the anticonvulsant effects in rats and mice is much more moderate than in humans (15-20% reduction in animals compared to >50% reduction in most human trials); the anticonvulsant effects of the KD are incomplete and of limited duration in many animal studies; the KD does not seem to diminish severity once seizure have began, and may even exacerbate them, owing to increased energy stores as a result of the KD.<sup>103</sup> These generalizations are similar to what we observed in this study. Therefore the lack of effect of the KD might be due to the limited efficacy in rat models overall - in which the current MSO rat study is included.

Another possible explanation might be that the KD is effective in some types of refractory epilepsies, but not in TLE. At the Mayo clinic the diet was traditionally thought to be useful only in the treatment of “idiopathic” epilepsies.<sup>104</sup> This was based on clinical experience of the author, and he gives no evidence to support why this would be

the case. But finding definitive evidence of the KD in the treatment of TLE is a challenge, as most data come from patients with generalized seizures. In a trial of the efficacy of the KD in generalized versus focal seizures, the diet was found to be equally effective in both.<sup>59</sup> However, only 34 focal patients were included in the study (compared to 100 generalized), outcomes tended to be better in younger patients, and focal epilepsies were not further delineated into subtypes. In the pediatric randomized control trial of the KD, 57 children had focal epilepsy (27 with structural abnormalities, 16 “presumed focal”, and 14 multifocal) and the KD was as effective for them as it was for patients with generalized epilepsy.<sup>55</sup> No patient subgroups of TLE were identified however, so it is hard to extrapolate these results specifically to TLE.

Another factor to take into consideration is that a lack of significant difference does not rule out the presence of a real, but small difference that cannot be detected due to the statistical power of the present study. With  $\alpha = 0.05$ ,  $\beta \geq 0.8$ , a 30% expected reduction in seizures, a standard deviation of 125%, and a two group comparison using a two-way student’s t-test, a minimum number of  $n = 16$  animals per group is required to detect a therapeutic effect. With our  $N$  of 12 KD animals, we are slightly below this number so it is possible that the KD is having an effect, it is just not detectable with the present number of animals.

One of the barriers to more widespread use of the KD, especially in adults is tolerability of the diet. While it is relatively easy to initiate the diet in infants and small children, few adolescents or adults are willing to cut carbohydrates completely out of their diet. Dietary guidelines must be followed strictly, however, for the diet to be effective and breaking ketosis diminishes the therapeutic effect of the diet almost

immediately.

The idea of food as therapy has been with us since antiquity, but one of the most dramatic examples was the ingestion of large amounts of liver to treat pernicious anemia, a previously fatal disease. Murphy, during his Nobel lecture in 1934 for, “Discoveries Concerning Liver Therapy in Cases of Anemia” said, “... [a] problem which, during the past few years, has particularly interested me, as a practitioner of medicine, has been the practical one of making treatment more bearable for the victim... who must necessarily continue treatment indefinitely in order to maintain a satisfactory state of health”.<sup>105</sup> This remains a laudable goal today and is particularly relevant to patients with intractable epilepsy on a ketogenic diet who must eat an exorbitant amount of fat while strictly limiting carbohydrate intake.

Distilling the essential aspects of the KD into a more bearable treatment will require an understanding of the mechanism of action of the diet. We are getting closer to an understanding, but we still do not know how the diet works. Further exploration, perhaps with different animal models will be needed to flesh this out. The KD was unsuccessful in this study, but there is good evidence that it is effective in humans, especially children with drug resistant epilepsy. Perhaps further research into the diet will yield novel treatments for those who need it most.

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## Chapter 7: Figure References and Legends

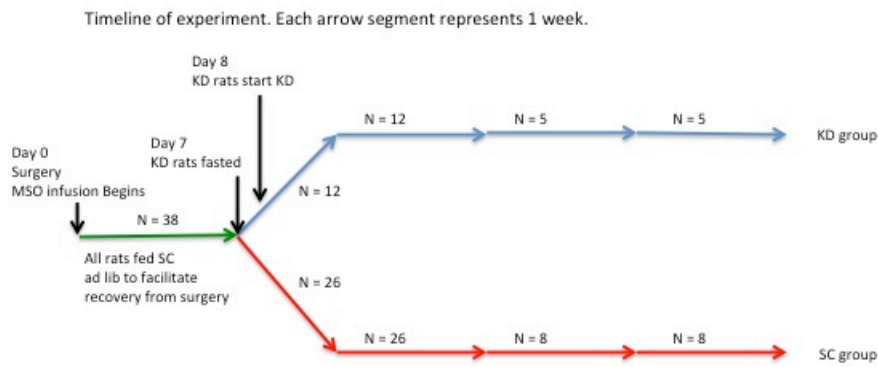


Figure 1. Timeline of the experiment. Each arrow segment represents 1 week of time.



Fig 3. Plot of the seizure frequency for all animals for the first seven days after MSO infusion. The line drawn through the boxes represents the average seizure frequency for that particular day. Note the cluster of initial seizures during days 1-2, followed by a decline in seizure frequency during days 3-5, with subsequent smaller increase during days 6-7.

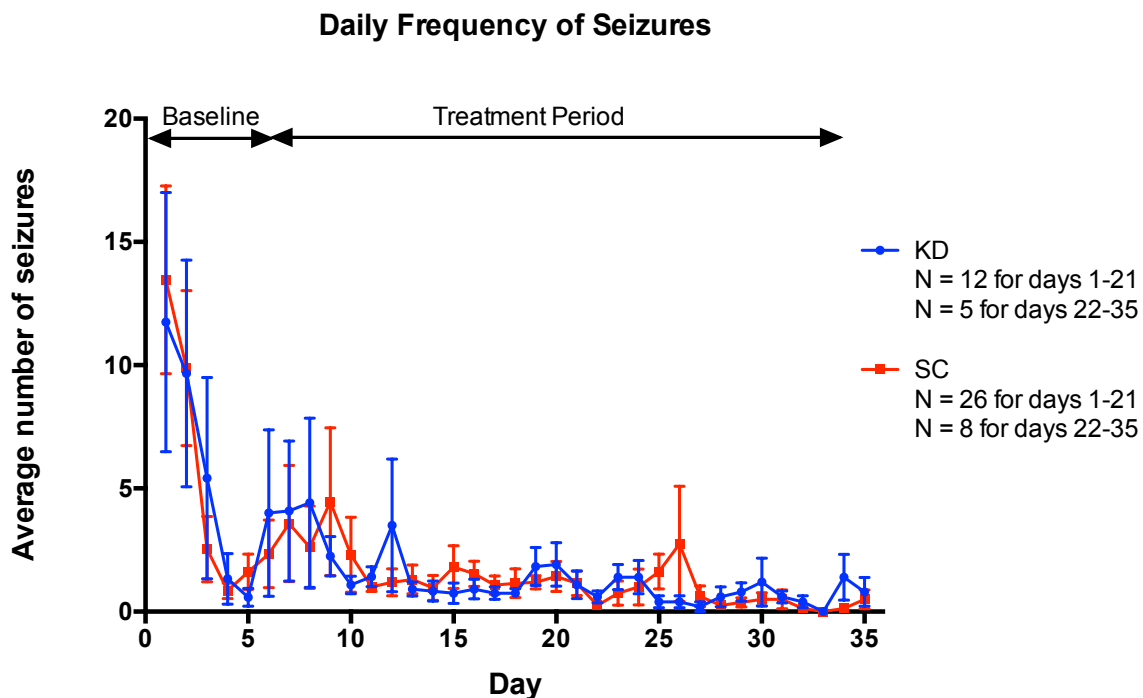


Fig 4. Average number of seizures each day for both groups of animals. The greatest number of seizures for both groups occurred during the first 3 days of the study; there is a nadir around day 5, then another peak days 6-9, followed by a slow taper



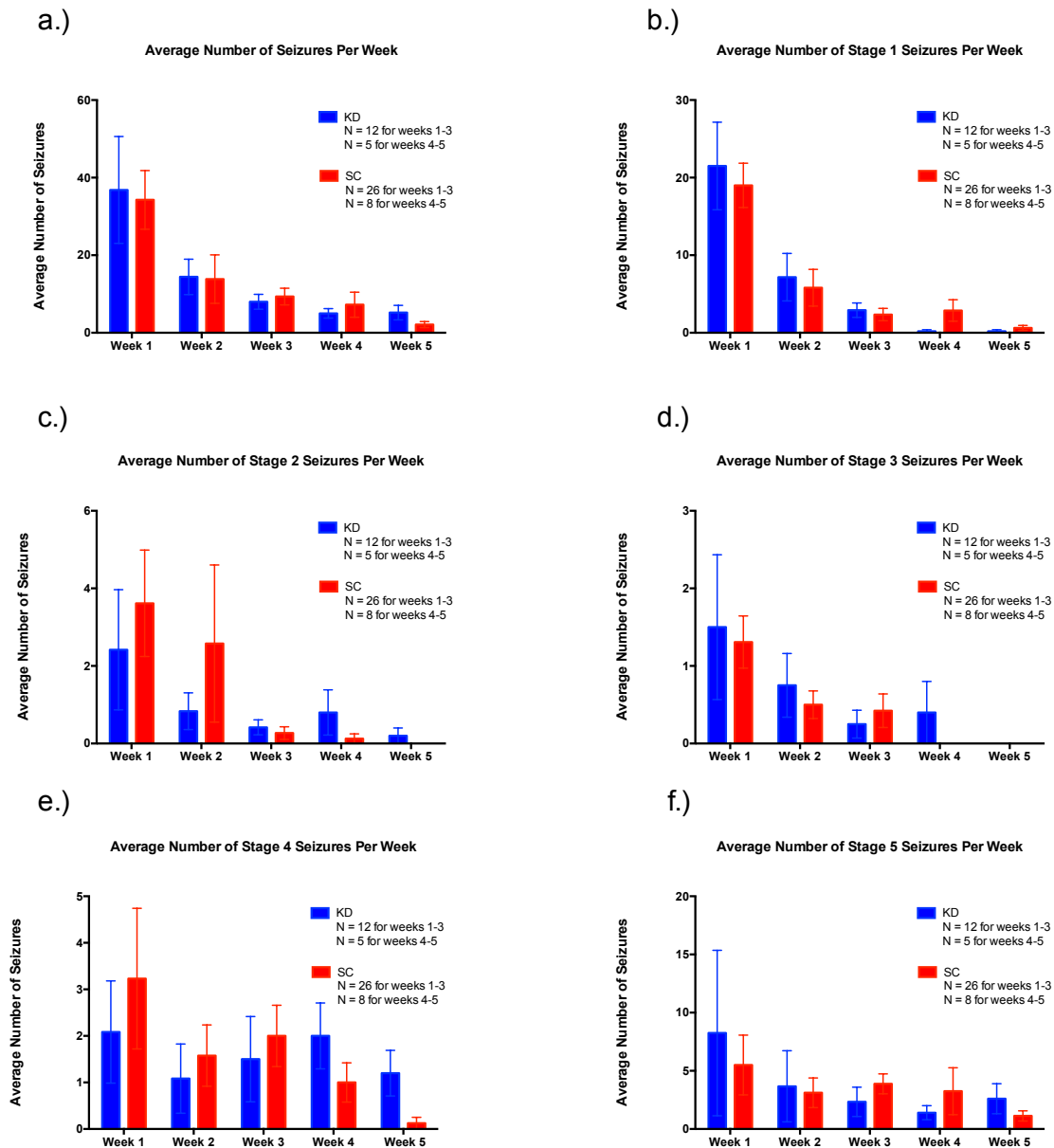


Fig. 5. Weekly frequency of all seizure types (a) and of seizure types at different behavioral severities (b-f) in KD treated (blue bars) and standard chow (SC, red bars) rats. All rats were given SC during week 1. KD started on week 2. Shown is the mean number of seizures. There were no significant differences in seizure frequency or severity between KD versus SC treated rats.

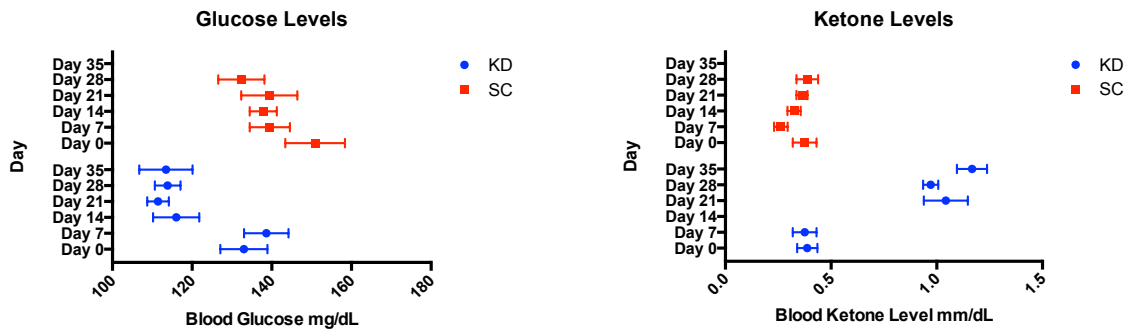


Figure 6. Blood glucose and ketone levels for a random selection of 8 rats in the KD and SC treated groups. Both groups were fed standard chow ad libitum for 7 days to facilitate recovery from surgery. The diet group started treatment on day 8. The first measurement taken while the rats were on the KD was on day 14. KD rats had significantly decreased blood glucose levels on days 14, 21, and 28. KD rats had significantly increased blood ketone levels on days 21 and 28.

## Chapter 8: Tables

Breakfast	Lunch	Dinner
2 medium eggs	2 hot dogs	Chicken 1.5 ounces
1 sausage patty	2 lettuce leaves, 1 thin slice tomato	½ cup green beans, 2 tsp butter
11 tsp butter	3.5 tbs mayonnaise	4 tbl vegetable oil
3 tbs heavy whipping cream		

Table 1. Sample ketogenic diet for 1 day (from Sirven et. al)

Diet	kcal/g	% Protein	% Fat	% Carbs
Standard Chow	3.3	20	13	67
Ketogenic Diet	7.1	8.7	89.2	2.1

Table 2. Comparison of macronutrients of standard chow vs. KD

	KD: mean # of all seizures ± SEM	SC: mean # of all seizures ±SEM	p-value
Week 1	36.8 ± 13.8	34.3 ± 7.6	0.86
Week 2	14.4 ± 4.6	13.8 ± 6.3	0.95
Week 3	8.0 ± 1.9	9.3 ± 2.1	0.70
Week 4	5.0 ± 1.2	7.3 ± 3.2	0.61
Week 5	5.2 ± 1.8	2.1 ± 0.8	0.11

Table 3. Comparison of the average number of seizures for KD vs. standard chow (SC) treated rats. The number of seizures were binned in one week intervals. During week 1, all rats are fed SC ad libitum. Treatment with the KD began during week 2. Student's t-test showed no significant reduction of seizures in any week with statistical significance determined using the Holm-Sidak method, with alpha = 5%.

	KD: mean # of seizures $\pm$ SEM	SC: mean # of seizures $\pm$ SEM	p-value
	<b>Stage 1</b>	<b>Stage 1</b>	
Week 1	21.5 $\pm$ 5.6	19.0 $\pm$ 2.9	0.66
Week 2	7.2 $\pm$ 3.1	5.8 $\pm$ 2.4	0.74
Week 3	2.9 $\pm$ 0.9	2.3 $\pm$ 0.8	0.67
Week 4	0.2 $\pm$ 0.2	2.8 $\pm$ 1.4	0.16
Week 5	0.2 $\pm$ 0.2	0.6 $\pm$ 0.3	0.36
	<b>Stage 2</b>	<b>Stage 2</b>	
Week 1	2.4 $\pm$ 1.5	3.6 $\pm$ 1.3	0.60
Week 2	0.8 $\pm$ 0.5	2.6 $\pm$ 2.0	0.57
Week 3	0.4 $\pm$ 0.2	0.3 $\pm$ 0.2	0.59
Week 4	0.8 $\pm$ 0.6	0.1 $\pm$ 0.1	0.18
Week 5	0.2 $\pm$ 0.2	0.0 $\pm$ 0.0	0.22
	<b>Stage 3</b>	<b>Stage 3</b>	
Week 1	1.5 $\pm$ 0.9	1.3 $\pm$ 0.3	0.81
Week 2	0.8 $\pm$ 0.4	0.5 $\pm$ 0.2	0.52
Week 3	0.3 $\pm$ 0.2	0.4 $\pm$ 0.2	0.62
Week 4	0.4 $\pm$ 0.4	0.0 $\pm$ 0.0	0.23
Week 5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	n/a
	<b>Stage 4</b>	<b>Stage 4</b>	
Week 1	2.1 $\pm$ 1.1	3.2 $\pm$ 1.5	0.63
Week 2	1.1 $\pm$ 0.7	1.6 $\pm$ 0.7	0.66
Week 3	1.5 $\pm$ 0.9	2.0 $\pm$ 0.7	0.67
Week 4	2.0 $\pm$ 0.7	1.0 $\pm$ 0.4	0.21
Week 5	1.2 $\pm$ 0.5	0.1 $\pm$ 0.4	0.02
	<b>Stage 5</b>	<b>Stage 5</b>	
Week 1	8.2 $\pm$ 7.1	5.5 $\pm$ 2.6	0.65
Week 2	3.7 $\pm$ 3.1	3.1 $\pm$ 1.3	0.84
Week 3	2.3 $\pm$ 1.3	3.8 $\pm$ 0.9	0.32
Week 4	1.4 $\pm$ 0.6	3.3 $\pm$ 2.0	0.50
Week 5	2.6 $\pm$ 1.3	1.1 $\pm$ 0.4	0.22

Table 4. Mean number of seizure for KD and SC rats sorted by severity.

	KD: Mean Blood Glucose Levels mg/dL $\pm$ SEM	SC: Mean Blood Glucose Levels mg/dL $\pm$ SEM	p-value
Day 0	133 $\pm$ 5.9	151 $\pm$ 7.5	0.08
Day 7	138 $\pm$ 5.6	140 $\pm$ 5.0	0.91
Day 14	116 $\pm$ 5.8	137 $\pm$ 3.4	0.005*
Day 21	111 $\pm$ 2.7	139 $\pm$ 7.0	0.004*
Day 28	114 $\pm$ 3.2	132 $\pm$ 5.8	0.019*
Day 35	113 $\pm$ 6.7	No Data	N/A

Table 5. Mean blood glucose levels for KD and SC rats. Significant differences are marked with an asterisk

	KD: Mean Blood Ketone Levels mm/dL $\pm$ SEM	SC: Mean Blood Ketone Levels mm/dL $\pm$ SEM	p-value
Day 0	0.39 $\pm$ 0.05	0.38 $\pm$ 0.06	0.87
Day 7	0.38 $\pm$ 0.06	0.26 $\pm$ 0.03	0.10
Day 14	No Data	0.33 $\pm$ 0.03	N/A
Day 21	1.04 $\pm$ 0.10	0.36 $\pm$ 0.03	< 0.0001*
Day 28	0.97 $\pm$ 0.04	0.39 $\pm$ 0.05	<0.0001*
Day 35	1.17 $\pm$ 0.07	No Data	N/A

Table 6. Mean blood ketone levels for KD and SC rats. Significant differences are marked with an asterisk