CARNITINE STATUS OF CHILDREN TREATED WITH THE KETOGENIC DIET FOR INTRACTABLE EPILEPSY

By

Jane E. Riebold

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CERTIFICATE OF APPROVAL

This is to certify that the Master's thesis of

Jane E. Riebold

has been approved

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Dr. Diane Stadler, PhD, RD, LD

_____

Dr. Melanie Gillingham, PhD, RD, LD

_____

Dr. Carter Wray, MD

_____

Ms. Pamela Haskell, RD, CSP, LD

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ATP	Adenosine triphosphate
AED	Antiepileptic drug
BCAA	Branched-chain amino acid
C2	Acetylcarnitine
C3	Propionylcarnitine
C4	Isobutyrylcarnitine/Butyrylcarnitine
C4-DC	Methylmalonylcarnitine
C4-OH	3-Hydroxybutyrylcarnitine
C5	Isovalerylcarnitine/2-Mebutyrlcarnitine
C5-DC	Glutarylcarnitine
C5-OH, 3-OH	3-Hydroxyisovalerylcarnitine
C5:1	Tiglylcarnitine/Methylcrotonylcarnitine
C6	Hexanoylcarnitine
C6DC	Adipoylcarnitine
C6-OH	3-Hydroxyhexanoylcarnitine
BzCn	Benzoylcarnitine
C8	Octanoylcarnitine
C8-DC	Suberylcarnitine

C8:1	Octenoylcarnitine
C10	Decanoylcarnitine
C10:1	Decenoylcarnitine
C10:2	Decadienoylcarnitine
C12	Dodecanoylcarnitine
C12:1	Dodecenoylcarnitine
C12-OH	3-Hydroxydodecanoylcarnitine
C14	Tetradecanoylcarnitine
C14:1	Tetradecenoylcarnitine
C14:2	Tetradecadienoylcarnitine
C14:OH	3-Hydroxytetradecanoylcarnitine
C14:1-OH	3-Hydroxytetradecenoylcarnitine
C16	Palmitoylcarnitine
C16-DC	Dicarboxypalmitoylcarnitine
C16:1	Palmitoleylcarnitine
C16-OH	3-Hydroxypalmitoylcarnitine
C16:1-OH	3-Hydroxypalmitoleylcarnitine
C18	Stearoylcarnitine
C18:1	Oleylcarnitine
C18:1-DC	Dicarboxyliccarnitine
C18:2	Linoleylcarnitine
C18-OH	3-Hydroxystearoylcarnitine

C18:1-OH	3-Hydroxyoleylcarnitine
C18:2-OH	3-Hydroxylinoleylcarnitine
CACT	Carnitine-acylcarnitine translocase
CoA	Coenzyme A
CPT-1	Carnitine-palmitoyltransferase 1
CPT-2	Carnitine-palmitoyltransferase 2
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
FAB-MS	Fast atom bombardment mass spectrometry
GC/MS	Gas chromatography mass spectrometry
GEE	Generalized estimating equation method
HIPAA	Health Insurance Portability and Accountability Act
HPLC	High performance liquid chromatography
IRB	Institutional Review Board
KD	Ketogenic diet
L-carnitine	Levocarnitine
LCFA	Long-chain fatty acid
MCT	Medium-chain triglyceride
OHSU	Oregon Health & Science University
REA	Radioenzymatic assay
TCA	Tricarboxylic acid
LCT	Long-chain triglyceride

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ABSTRACT

Background

The ketogenic diet (KD) is a very high fat and low carbohydrate diet that reduces seizure frequency in some children with epilepsy. The KD may increase risk for deficiency of carnitine, a nutrient required for fatty acid oxidation and ketone production. Although carnitine is supplemented during KD therapy by many medical centers, supplemental carnitine may be cost-prohibitive, poorly tolerated, and possibly unnecessary. This study aimed to evaluate: 1) the impact of the KD on plasma carnitine concentrations, 2) possible correlations between plasma carnitine and β-hydroxybutyrate concentrations, and 3) the association between plasma carnitine concentration and reduction in seizure frequency and number of antiepileptic drugs (AEDs) prescribed.

Methods

We reviewed medical records for pediatric patients at Doernbecher Children's Hospital (Portland, OR) who were treated by the KD (3:1 ratio or higher) for epilepsy who were not prescribed supplemental carnitine during KD therapy. Mean plasma concentrations of free carnitine, acylcarnitine, total carnitine, and acyl/free carnitine concentration ratio were calculated before and 1, 3, and 5 – 8 months after KD initiation. Mean plasma concentrations of individual acylcarnitine species were

calculated before and 5 – 8 months after KD initiation. Difference in means was assessed, linear regression was performed, and odds ratios were calculated.

Results

Medical records of 22 patients were analyzed (mean age 3.9 ± 4.8 years, range 0.08 – 16.0 years). Mean plasma free carnitine was lower than baseline at 1, 3, and 5 — 8 months after KD initiation (not statistically significant). Mean plasma concentration of total carnitine was significantly higher than baseline at 1 and 5 — 8 months after KD initiation. Mean plasma concentration of acylcarnitine and acyl/free carnitine concentration ratio were significantly higher than baseline at 1, 3, and 5 — 8 months after KD initiation. Mean plasma concentrations were significantly higher than baseline at 5 – 8 months after KD initiation for the acylcarnitine species: C2, C4-OH, C8, C10, C10:1, C14:1, C18 – C18:2. Plasma β-hydroxybutyrate concentration was significantly negatively correlated with plasma concentration of free ($p=0.00$) and total ($p<0.01$) carnitine, and significantly positively correlated with plasma acyl/free carnitine concentration ratio ($p=0.01$) and plasma concentrations of C2 ($p=0.04$) and C3 ($p<0.01$). Participants with a plasma acyl/free carnitine concentration ratio of at least 1.5 were 10 times more likely to achieve at least a 50% reduction in seizure frequency ($p<0.05$). No statistically significant odds ratios were observed for likelihood of withdrawal of at least one AED.

Discussion and Conclusion

The KD significantly impacted mean plasma carnitine concentrations for pediatric patients who were not prescribed supplemental carnitine. We observed increased: plasma acylcarnitine and total carnitine concentrations, acyl/free carnitine

concentration ratio, and concentrations of certain acylcarnitine species. Degree of ketosis was higher for children with: lower plasma concentrations of free and total carnitine, higher plasma acyl/free carnitine concentration ratio, and higher plasma concentrations of C2 and C3. Children with plasma acyl/free carnitine concentration ratios of at least 1.5 may be more likely to experience reduction in seizure frequency. We found insufficient evidence to suggest that AED withdrawal was influenced by plasma carnitine concentration.

CHAPTER 1

Introduction

Epilepsy is a neurological condition that affects approximately 1% of children in the United States (1). Over 345,000 children in the United States currently have a diagnosis of epilepsy, including more than 2,500 children in the state of Oregon (2). Adult and childhood epilepsy represents a combined direct (medical) and indirect (lost or reduced earnings and productivity) cost of $15.5 billion annually (2). An estimated 10 – 23% of children with epilepsy experience intractable epilepsy, meaning that their condition is not well controlled despite attempted treatment with two or more antiepileptic drugs (AEDs) (3,4). About 50% of children with intractable epilepsy respond positively (defined as at least 50% reduction in seizure frequency and/or reduced number of AEDs required) to treatment with the ketogenic diet (5-7).

The ketogenic diet is a very high fat and extremely low carbohydrate therapeutic diet that is primarily used to treat intractable seizure disorders of various etiologies (5). The Mayo Clinic first employed the ketogenic diet as a treatment for pediatric epilepsy in the early 1920s, and it continues to be implemented today in many major medical centers worldwide (8,9). Classic ketogenic diets are typically designed to provide 3 – 4 g of fat for every 1 g of protein and carbohydrate combined (i.e., 87 – 90% of total energy intake from fat) (5). The ketogenic diet is intended to mimic the metabolic state of

prolonged fasting in order to induce ketosis, a condition during which blood and tissue concentrations of ketones become very high. Although the underlying anticonvulsant mechanism of the ketogenic diet is unknown, efficacy is attributed to the change from predominantly glucose-based energy production to predominantly ketone-based energy production for most body tissues and particularly for the brain. Ketogenic diet therapy has been shown to result in long-term and lasting reductions in both seizure frequency and AED usage even after dietary treatment is discontinued (10).

Identifying adjunct therapies that could enhance tolerance to or efficacy of the ketogenic diet would have a significant public health impact by producing better seizure management and better quality of life for pediatric patients. Carnitine supplementation for children treated with the ketogenic diet may be one such treatment, as supplemental carnitine has been shown to accentuate the rise in fasting blood ketone concentration for patients with other chronic disorders (11). If elevated blood ketone concentration plays a role in the anticonvulsant activity of the ketogenic diet, and if carnitine supplementation enhances synthesis of ketones, then carnitine supplementation during ketogenic diet therapy may result in further reduction in seizure frequency and AED requirements for children with intractable epilepsy.

Despite the potential benefits of supplemental carnitine during ketogenic diet therapy, there is no clinical consensus on:

1) whether the ketogenic diet impacts carnitine status,
2) if carnitine supplementation improves response to the ketogenic diet, and
3) what dose of supplemental carnitine would optimize the therapeutic impact.

Protocols regarding carnitine supplementation for children treated with the ketogenic diet vary widely between institutions. Some programs prescribe pharmacological doses of carnitine up to 100 mg/kg/day or more, while other programs do not prescribe carnitine at all for children who initiate or maintain ketogenic diet treatment. Clinicians and parents who do choose to supplement with carnitine for pediatric patients treated with the ketogenic diet anecdotally report "improved well-being, energy levels and seizure control" independent of baseline carnitine concentration before supplementation (12). This clinical variability has generated a clear need for future studies examining the potential requirement for carnitine supplementation in these patients.

As a first step to address this gap in the knowledge, we conducted a medical record review to examine the impact of the ketogenic diet on plasma carnitine concentration in children with intractable epilepsy and to assess the potential associations between plasma carnitine concentration and response to ketogenic diet therapy.

CHAPTER 2

Background

Epilepsy in the pediatric population

Epilepsy is a neurological condition that is diagnosed after the occurrence of two or more unprovoked seizures (13). Seizures are periods of altered brain activity that result in distorted awareness or sensations, behavior changes, or involuntary muscular activity (13). Seizures are typically as brief as a fraction of a second or as long as several minutes.

Epilepsy affects over 345,000 children in the United States (2). Intractable epilepsy occurs when seizures are not well controlled by AEDs, meaning that the treatment course is complicated by recurrent seizures or periods of break-through seizing. Approximately 10 – 23% of children who are diagnosed with epilepsy experience intractable epilepsy (3,4). An estimated 50% of these patients respond positively to the ketogenic diet with at least a 50% reduction in seizure frequency and/or reduced number of AEDs required (5-7).

The ketogenic diet: classic, MCT, modified Atkins, and low glycemic index

The ketogenic diet is defined as a very high fat and extremely low carbohydrate diet with a primary clinical indication for the treatment of intractable seizure disorders of various etiologies (5). The ketogenic diet is intended to mimic the metabolic state of

prolonged fasting in order to induce ketosis, a condition during which blood and tissue concentrations of ketones become very high. Historically, the ketogenic diet has also been initiated with a period of restricted caloric intake (75% of estimated needs) and fluid intake (80% of estimated needs) to hasten the achievement of ketosis, although such strict limitations are no longer deemed necessary by many medical centers (14,15).

The classic ketogenic diet is typically designed to provide 3 – 4 g of fat for every 1 g of protein and carbohydrate combined (i.e., 87 – 90% of total energy intake from fat) (5). The dietary prescription is usually represented as a ratio of grams fat to combined grams of protein and carbohydrate (e.g., 3:1 or 4:1). The ketogenic diet can be also modified to provide only 2 g of fat for every 1 g of protein and carbohydrate combined (i.e., 2:1) when a higher dietary protein or carbohydrate content is desired.

The fat consumed in the classic ketogenic diet predominantly consists of long chain triglycerides (LCTs), which are the type of fats typically present in foods. The diet can also be prescribed with medium chain triglycerides (MCTs) as the primary source of fat (5). Both the classic ketogenic diet and the MCT-based ketogenic diet have been proven effective for the treatment of epilepsy (5). Advantages of the MCT-based ketogenic diet include greater ketone yield per kilocalorie of energy consumed and increased ease of dietary fatty acid absorption (5). This is because MCTs are taken up from the gut epithelium and transported directly through portal circulation to the liver for repackaging and distribution, rather than travelling through the lymphatic system first as LCTs do. The greater relative yield of ketones from MCTs also requires a slightly smaller proportion of the total energy consumed be allotted to dietary fat, leaving more

room for protein and carbohydrate in the diet and therefore providing improved palatability. Unfortunately, MCT oil is expensive, and participants undergoing MCT ketogenic therapy often experience significant gastrointestinal side effects. This often leads to difficulty tolerating the MCT-based ketogenic diet.

The modified Atkins diet is a further liberalized version of the ketogenic diet. The modified Atkins diet utilizes a lower ratio of grams fat to grams of protein and carbohydrate combined (roughly 1:1) and does not require restriction of protein, energy, or fluids. The modified Atkins diet can therefore be more easily tolerated for longer periods of time by some patients if it provides acceptable seizure control (16).

The low-glycemic index diet is the least restrictive type of dietary therapy and restricts dietary carbohydrate to 40 – 60 grams per day, preferably from foods with a glycemic index of less than 50. Glycemic index is calculated as the area under the blood glucose curve after ingestion of a particular food, with pure ingested glucose indexed to 100 (17).

Benefits and efficacy of ketogenic dietary therapy

The ketogenic diet has been shown to improve seizure control many times since its development in the 1920's, with ketogenic diet therapy typically resulting in at least a 50% reduction in seizure frequency for about half of patients within the first 3 – 6 months of treatment (5,7). A randomized, controlled clinical trial of 145 pediatric patients age 2 – 16 years demonstrated that 3 months of ketogenic diet therapy reduced seizure activity to 62% of baseline compared to an increase to 137% of baseline observed in the control group (5). Participants included in this study had previously

failed to achieve seizure control despite attempted treatment with at least two AEDs and experienced seizures either daily or a minimum of 7 times per week prior to ketogenic diet initiation.

An additional benefit of ketogenic dietary therapy is that it does not contribute to the medication burden of patients with epilepsy and may actually allow for the partial or complete withdrawal of previously required AEDs. Reduction in AEDs (either of the total number of medications or of individual medication dosage) may be possible as soon as 1 month after initiating the ketogenic diet (18). Withdrawal of AEDs is desirable due to the many known adverse effects of AED therapy in children, which include: nystagmus, decreased bone density, hepatic dysfunction, hematological changes, neuropathy, gingival hypertrophy, skin rashes, nutrient deficiencies (e.g., folate, vitamin B6, vitamin B12, and vitamin D) and changes in cognition, weight, and behavior (19-25).

The ketogenic diet has also been shown to result in long-term and lasting reductions in seizure frequency and AED use even after discontinuation of ketogenic diet therapy (10). A long-term, observational study tracked 150 children with epilepsy who were treated with ketogenic diet therapy. The percentage of the 150 participants who continued on ketogenic diet therapy at select time points was described as: 12 months (55%), 2 years (39%), 3 years (20%), 4 years (13%), and up to 6.5 years (11%) (10). Children who remained on the ketogenic diet for at least 1 year (n=83) were surveyed via questionnaire or telephone conversation at 3 – 6 years after ketogenic diet initiation. At the time of survey, 24% of the children were seizure-free, 25% experienced a 90 – 99% decrease in seizure activity, and 29% experienced a 50 – 90% decrease in

seizure activity. With respect to AED reduction at the time of survey, 34% did not require any AEDs and 37% only required one AED (compared to 7% and 34% at ketogenic diet initiation, respectively). These positive effects on seizure frequency and AED requirements were observed despite the fact that only 2 of the 83 children still adhered to the ketogenic diet at the time of survey (10).

Undesirable effects of the ketogenic diet

An observational study of 129 patients enrolled in ketogenic diet therapy from July 1995 to October 2001 identified the following early-onset side effects (occurring within the first 4 weeks of ketogenic diet initiation): dehydration (especially when initiation included a fluid restriction), nausea, vomiting, diarrhea, constipation, and occasional gastritis or fat intolerance. Less common early-onset side effects (ordered by frequency of patient report) included: hypertriglyceridemia, transient hyperuricemia, hypercholesterolemia, various infectious diseases, symptomatic hypoglycemia, hypoproteinemia, hypomagnesemia, repetitive hyponatremia, low concentration of high-density lipoprotein, lipoid pneumonia due to aspiration, hepatitis, acute pancreatitis, and persistent metabolic acidosis. Late-onset side effects (occurring later than 4 weeks after ketogenic diet initiation) included: osteopenia, renal stones, cardiomyopathy, secondary hypocarnitinemia, and iron deficiency anemia. Most of these complications were seen to be transient; however, 17% of patients in the study experienced such significant side effects that they withdrew from ketogenic diet treatment (26).

An overview of carnitine

Carnitine is a water-soluble, vitamin-like nutrient and amino acid derivative. Levocarnitine (L-carnitine) is the biologically active isomer of carnitine (27). Carnitine

can be obtained exogenously from the diet and is also synthesized endogenously from amino acids. Carnitine is not normally considered an essential nutrient (meaning it does not need to be consumed regularly in the diet for good health). However, carnitine can become conditionally essential (meaning that it must be consumed in the diet during specific situations only) for children treated with the ketogenic diet (28).

Endogenous synthesis of carnitine occurs in the liver and kidneys and requires the essential amino acids lysine and methionine as substrate. The rate-limiting factor for carnitine synthesis is the availability of methylated lysine residues that are released during lysosomal breakdown of body proteins (e.g., skeletal muscle, cellular proteins, etc.) (29). Carnitine synthesis also requires the micronutrient cofactors of vitamin C, vitamin B6, and iron (29).

Dietary carnitine is obtained almost exclusively from foods of animal origin. Depending on the amount ingested, between 54 – 87% of dietary carnitine is absorbed from the small intestine into the enterocyte via both passive diffusion and active, sodium-dependent cotransport (30). It has been hypothesized that diffusion into the enterocyte occurs more readily for esterified sources of carnitine than for carnitine in the free (unesterified) form (30). Dietary carnitine that is not absorbed is degraded to γ-butyrobetaine and trimethylamine by microbes in the large intestine; γ-butyrobetaine is then excreted in the feces, while trimethylamine is transported into the blood, filtered out by the kidneys, and subsequently excreted in the urine (31).

Adults eating a mixed diet that includes red meat and other animal products typically consume about 60 – 180 mg of carnitine per day and synthesize about 25% of their total body carnitine stores (20). Vegans consume considerably less carnitine (about

10 – 12 mg per day) due to their avoidance of animal-derived foods. Vegans must synthesize about 90% of their total body carnitine stores (20). Vegetarians only have moderately reduced plasma free and total carnitine concentration (10 – 20% less than omnivorous adults) as a result of upregulated endogenous synthesis and increased renal reabsorption of carnitine; increased renal reabsorption may possibly occur as an effect of the reduced filtered load of carnitine that is associated with a lower fat diet (32,33). For infants, soy-based formulas and other non-milk formulas are now fortified with the amount of carnitine normally found in human milk (0.6 – 1.1 mg per 100 mL) (34,35).

Carnitine deficiency is rare in the general population and can result in consequences that range from mild to serious, including: generalized weakness, fatigue, reduced muscle strength, hepatitis, and cardiomyopathy (36,37). Primary carnitine deficiency results from a genetic defect that negatively impacts the cellular transport mechanism by which carnitine enters the cell. This leads to low intracellular free carnitine concentration, increased concentration of free carnitine in the blood, and subsequent excessive renal excretion of free carnitine. Primary carnitine deficiency is therefore observed as prolonged low (<5 μmol/L) blood concentration of free carnitine along with relatively low blood concentration of acylcarnitine and total carnitine (38).

Secondary carnitine deficiency occurs as a result of fatty acid overload of the mitochondrial transport system, leading to markedly increased concentrations of acyl-CoA groups in the mitochondria. Acyl-CoA groups must then be esterified to available free carnitine and are subsequently transported out of the mitochondria in the form of acylcarnitine. Acylcarnitine is excreted by the kidneys in large amounts when present in

high concentrations. Secondary carnitine deficiency is therefore characterized by an uncommonly high ratio of acyl/free carnitine concentrations, as well as relatively low concentrations of free carnitine, acylcarnitine, and total carnitine (39). Circulating carnitine concentration may also be reduced independent of genetic abnormalities or metabolic stress by reduced endogenous carnitine synthesis during protein-calorie malnutrition, or by an inadequate supply of the cofactor micronutrients (i.e., vitamin C, vitamin B6, and iron) needed for endogenous synthesis (40,41).

A closer look at fat metabolism

Under normal metabolic conditions, glucose is the primary fuel for almost all tissues in the human body and particularly for the brain. Energy is produced primarily through glycolysis, a process that breaks down glucose to pyruvate to yield adenosine triphosphate (ATP). Additional energy can be generated by activation of the tricarboxylic acid (TCA) cycle and the electron transport chain.

During extended periods of fasting or inadequate carbohydrate intake, glycolysis is greatly reduced and activation of the TCA cycle is limited due to reduced availability of oxaloacetate, a crucial TCA cycle intermediate that is preferentially used for gluconeogenesis under hypoglycemic conditions. Although glycolysis and activation of the TCA cycle continue to some extent, the primary source of energy soon becomes fatty acid oxidation, either of ingested triglycerides or of fatty acids liberated from adipose tissue through lipolysis. Fatty acids liberated from adipose tissue are transported in the blood loosely bound to the protein albumin until they reach the liver, where they are taken up by hepatocytes and oxidized for energy. Ingested triglycerides

are consumed either as LCTs (most of the fat consumed in the typical American diet) or as MCTs. Dietary LCTs and MCTs are both absorbed by enterocytes that line the small intestine. MCTs are more water-soluble than LCTs and are therefore able to diffuse directly into the enterocyte without encountering digestive enzymes, after which they are transported directly to the liver via the portal vein. In contrast, LCTs are first emulsified by bile acids in the small intestine in order to form micelles, which are then taken up by the enterocyte and repackaged to form chylomicrons. Chylomicrons are exported into the lymphatic system for transport, where they eventually travel to the left subclavian vein and enter blood circulation via the thoracic duct. Chylomicrons then circulate to the liver and are finally taken up by hepatocytes.

Ketone synthesis and utilization

Once fatty acids derived from lipolysis and dietary triglycerides are absorbed by hepatocytes, they are rapidly converted within the cytosol to acyl-CoA derivatives (the product of a fatty acid molecule that has been esterified to coenzyme A) by the enzyme acyl-CoA synthase. These acyl-CoA derivatives are transported into the matrix of the mitochondria via the carnitine shuttle and are oxidized for energy via β-oxidation. β-oxidation is the process of degrading fatty acid chains to generate acetyl-CoA units, which then enter the TCA cycle to produce ATP.

When the TCA cycle is not able to utilize excessive amounts of acetyl-CoA that are generated, such as during times of fasting or inadequate carbohydrate intake, acetyl-CoA does not enter the TCA cycle but is instead converted to ketone bodies. Ketone bodies are defined as water-soluble molecules that can be used for energy by

extra-hepatic tissues. The parent ketone body, acetoacetate, is synthesized in hepatic mitochondria and can be converted to β-hydroxybutyrate within the mitochondria by β-hydroxybutyrate dehydrogenase (42). Alternately, acetoacetate can spontaneously generate a third ketone body, acetone, via slow non-enzymatic decarboxylation (42). Although ketone bodies cannot be used by the liver to produce energy, they can be metabolized by other tissues and particularly by the brain (42).

Ketone bodies are released into circulation and enter the brain in proportion to the degree of ketosis, i.e. the overall concentration of circulating ketone bodies (42). Acetoacetate and β-hydroxybutyrate cross the blood-brain barrier via the monocarboxylic acid transporter, while acetone is able to freely diffuse across the blood-brain barrier (43). Expression of the monocarboxylic acid transporter is upregulated in response to increased circulating concentrations of acetoacetate and β-hydroxybutyrate (44). Ketone bodies that enter the brain are then converted to acetyl-CoA by D-β-hydroxybutyrate dehydrogenase, acetoacetate-succinyl-CoA transferase, and acetoacetyl-CoA-thiolase (42). Acetyl-CoA then enters the TCA cycle within cerebral mitochondria and results in the production of ATP. In this manner, ketosis produces an alternative method of energy generation for the brain.

The carnitine shuttle and β-oxidation of fatty acids

Adequate carnitine status is considered to be crucial to the success of the ketogenic diet. This is because carnitine transports long-chain fatty acids (LCFAs) across the mitochondrial membrane into the inner mitochondrial matrix for β-oxidation (defined previously in this text as the process of degrading fatty acid chains to yield

acetyl-CoA units). β-oxidation of fat creates acetyl-CoA, which may then enter the TCA cycle (during times of adequate carbohydrate intake) or be converted to ketone bodies (during times of inadequate carbohydrate intake).

LCFAs that have been converted to acyl-CoA in the cytosol are transported into the mitochondria via an intracellular system called the carnitine shuttle. The carnitine shuttle consists of three enzymes: carnitine-palmitoyltransferase 1 (CPT-1), carnitine-acylcarnitine translocase (CACT), and carnitine-palmitoyltransferase 2 (CPT-2). CPT-1 esterifies the acyl portion of acyl-CoA onto carnitine to produce esterified carnitine, called acylcarnitine. CACT transfers the acylcarnitine into the mitochondrial matrix. CPT-2 then esterifies the acyl portion of the acylcarnitine back onto to CoA to reproduce acyl-CoA and free carnitine within the mitochondrial matrix. At this point, acyl-CoA is finally able to enter the β-oxidation pathway in order to generate acetyl-CoA.

Carnitine also modulates free coenzyme A homeostasis. As acyl-CoA begins to accumulate within the mitochondrial matrix, short-chain acyl units are esterified onto free carnitine to produce acylcarnitine and free CoA; this process maintains sufficient free coenzyme A to participate in further enzymatic reactions within the mitochondria. Short-chain acylcarnitines are then transported back out of the mitochondria by CACT (45). Once outside of the mitochondrial matrix, the acyl unit may be esterified back onto free CoA to reproduce acetyl-CoA and free carnitine. Acetyl-CoA may then be oxidized in the TCA cycle or used for the synthesis of ketones. Therefore, carnitine contributes to hepatic synthesis of ketones, the presumed mediator of anticonvulsant activity of the ketogenic diet, through its role as a transporter of fatty acid units across the mitochondrial membrane.

Biochemical parameters for carnitine

Carnitine is present in all biological fluids in two major forms: free carnitine and acylcarnitine. Total carnitine is measured as the sum of free carnitine and acylcarnitine. The acylcarnitine synthesized during mitochondrial fatty acid metabolism can be further characterized as multiple unique acylcarnitine species according to the type of fatty acid chain that remains bound to the carnitine molecule. Acylcarnitine species range from 2 – 18 carbons in length and are named according to their molecular structure. Acetylcarnitine is the primary acylcarnitine species synthesized and excreted in the urine during fatty acid oxidation under normal metabolic conditions (46).

Carnitine concentrations are most easily measured in plasma, dried blood spots, dried bile spots, and urine (28). Relative concentrations in these tissues vary greatly, with more than 90% of body carnitine stores located in the muscle and only relatively minuscule amounts circulating in the blood (27). Relative concentrations and percentages of carnitine stores within body tissues are summarized in Table 1.

Table 1: Distribution of carnitine within primary storage sites for a 70 kg adult (31)

Tissue type	Carnitine concentration	Carnitine content	% Total body carnitine
Plasma	50 µmol/L	0.2 µmol	0.1%
Extracellular fluid + plasma	50 µmol/L	0.7 µmol	0.5%
Skeletal muscle	4200 µmol/kg	126 µmol	97%
Heart	1300 µmol/kg	0.5 µmol	0.4%
Kidney	600 µmol/kg	0.2 µmol	0.2%
Liver	1000 µmol/kg	1.5 µmol	1%
Brain*	---	---	---

*Carnitine stores and concentrations in the brain are currently not known

Historical assessment of carnitine

Established methods of carnitine assessment include: enzymatic assay, radioenzymatic assay, spectrophotometric assay, high performance liquid chromatography, gas chromatography mass spectrometry, fast atom bombardment mass spectrometry, and electrospray ionization tandem mass spectrometry (39,47,48). In the mid-1980's, enzymatic and radioenzymatic assays were primarily used to assess plasma free carnitine concentration. Plasma acylcarnitine concentration was assessed by high performance liquid chromatography. Plasma total carnitine concentration was calculated as the sum of plasma concentrations of free carnitine and all acylcarnitine species (48). The next method of carnitine assessment to become widely used was gas chromatography mass spectroscopy of hydrolyzed carnitine fatty acyl residues. Gas chromatography mass spectrometry was quickly followed by the development of fast atom bombardment mass spectrometry and electrospray ionization tandem mass spectrometry (ESI-MS/MS) (48).

Current assessment of carnitine

Currently, carnitine concentration is almost exclusively measured by ESI-MS/MS. Through the ESI-MS/MS method, free carnitine is assessed by non-derivatized MS/MS after addition of a fixed amount of stable, isotope-labeled free carnitine (49). Total carnitine is assessed by non-derivatized, base-catalyzed hydrolysis MS/MS also after the addition of stable, isotope-labeled carnitine (39,49). Acylcarnitine concentration is calculated as the difference between free and total carnitine concentrations. The ratio of acyl/free carnitine concentrations is an additional parameter for carnitine that is

calculated mathematically from the aforementioned data. Identification and measurement of individual acylcarnitine species concentrations is performed by derivatized hydrolysis to individual carnitine esters, addition of isotope-labeled standards, and ESI-MS/MS. Acylcarnitine species are identified individually by ion mass scanning and are quantified by comparison to abundance of the isotope-labeled internal standards (28). The comprehensive quantitative assessment of acylcarnitine species concentrations is often referred to as an acylcarnitine profile.

Interpretation of carnitine status

Free and total plasma carnitine concentrations and the ratio of acyl/free plasma carnitine concentrations are most useful for identification of carnitine deficiency, which has been historically defined as plasma free carnitine concentration less than 20 µmol/L or a ratio of plasma acyl/free carnitine concentration ratio of greater than 0.4 (37). In current practice, laboratories more often use age-specific reference ranges to categorize carnitine status. Additionally, an elevated ratio of acyl/free plasma carnitine concentrations may not always indicate deficiency, as it is also frequently seen in individuals who are in a state of ketosis. The ratio of plasma acyl/free carnitine concentrations has been postulated to indicate the strength of ketosis rather than possible deficiency for individuals treated with ketogenic diet therapy (36). Plasma acyl/free carnitine concentration ratios from 0.44 – 3.25 have been observed in children treated by the ketogenic diet (36). Relevant reference ranges for carnitine are described in Table 2.

Acylcarnitine profiles are clinically most useful for identification of organic

Table 2: Reference ranges for plasma carnitine concentration (ARUP Laboratories, Salt Lake City, UT) (50)

Parameter	Reference range (plasma)		
	1 – 12 months	**1 – 6 years**	**7 – 20 years**
Free carnitine (µmol/L)	29 – 61	25 – 55	22 – 63
Acylcarnitine (µmol/L)	7 – 24	4 – 36	3 – 38
Total carnitine (µmol/L)	38 – 73	35 – 90	31 – 78
Acyl/free carnitine	0.1 – 0.8	0.1 – 0.8	0.1 – 0.9

acidemias and fatty acid oxidation disorders, such as carnitine palmitoyltransferase I or carnitine palmitoyltransferase II deficiency, both of which are contraindications for ketogenic diet treatment. Acylcarnitine profiles can appear falsely normal in a subject with a fatty acid oxidation disorder in the setting of carnitine deficiency because there may be insufficient available carnitine to accumulate upstream of the metabolic block. For this reason, carnitine profiles performed for the identification of a metabolic abnormality should always be assessed in conjunction with free and total carnitine concentrations. Even slightly elevated concentrations of certain acylcarnitine species should be cause for concern when they occur simultaneously with low free and acylcarnitine concentrations. Although some laboratories may report free carnitine (denoted as C0) in the acylcarnitine profile, this means of measuring free carnitine concentration is less accurate because of the additional processing required in order to analyze acylcarnitine species within a single blood sample.

Acylcarnitine profiles are also useful for identification of ketosis (28). Ketosis is represented by an acylcarnitine profile that demonstrates elevated concentrations of the acylcarnitine species acetylcarnitine (C2) and 3-hydroxylbutyrlcarnitine (C4-OH)

(28). Conversely, otherwise normal acylcarnitine profiles characterized by increased plasma concentrations of the corresponding acylcarnitine species for specific fatty acid chain lengths and compositions may reflect either mobilization of fatty acids stored as adipose tissue during fasting (15 – 20 hours in duration) or ingestion of a high fat load by a metabolically normal individual (51).

Review of acylcarnitine profiles for diagnostic purposes should be performed by a board-certified clinical biochemical geneticist who is familiar with the complexities of acylcarnitine profiles and able to assess relevant patterns in acylcarnitine species concentrations compared to their reference ranges (28). Reference ranges for concentrations of acylcarnitine species are provided in Table 3.

Risk factors for carnitine deficiency

Patients prescribed the ketogenic diet are at greater risk for carnitine deficiency due to increased carnitine demand resulting from heightened rate of fatty acid oxidation. Additionally, urinary carnitine excretion may be increased. In a carnitine-sufficient individual who is consuming a normal diet, less than 16% of the carnitine filtered by the kidneys is excreted in the urine (52). However, urinary carnitine excretion in children treated by the ketogenic diet may be increased due to potentially higher glomerular-filtered load of acylcarnitine; this has been observed previously in individuals consuming a high- fat diet (33).

Another risk factor for carnitine deficiency specific to patients with epilepsy is their history of AED usage (36,53). AED regimens that include valproic acid are associated with low and potentially depleted blood and tissue carnitine concentrations

Table 3: Reference ranges for plasma acylcarnitine species concentrations (ARUP Laboratories, Salt Lake City, UT) (55)

Acylcarnitine species	Chemical name (as -carnitine)	Reference range (plasma) (µmol/L)	
		8 days – 7 years	≥8 years
C2	Acetyl-	3.69 – 24.71	3.74 – 16.56
C3	Propionyl-	0.00 – 0.97	0.00 – 0.83
C4	Isobutyryl-/butyryl-	0.00 – 0.50	0.00 – 0.45
C4-DC*	Methylmalonyl-	--	--
C4-OH*	3-hydroxybutyryl-	--	--
C5	Isovaleryl-/2-mebutyrl-	0.00 – 0.28	0.00 – 0.30
C5-DC	Glutaryl-	0.00 – 0.07	0.00 – 0.09
C5-OH, 3-OH	3-hydroxyisovaleryl-	0.00 – 0.07	0.00 – 0.07
C6DC*	Adipoyl-	--	--
C6-OH*	3-hydroxyhexanoyl-	--	--
C5:1*	Tiglyl-/methylcrotonyl-	--	--
C6	Hexanoyl-	0.00 – 0.12	0.00 – 0.12
BzCn*†	Benzoyl-	--	--
C8	Octanoyl-	0.00 – 0.23	0.00 – 0.23
C8-DC*†	Suberyl-	--	--
C8:1	Octenoyl-	0.00 – 0.63	0.00 – 0.61
C10	Decanoyl-	0.00 – 0.35	0.00 – 0.31
C10:1	Decenoyl-	0.00 – 0.41	0.00 – 0.31
C10:2*	Decadienoyl-	--	--
C12	Dodecanoyl-	0.00 – 0.12	0.00 – 0.12
C12:1	Dodecenoyl-	0.00 – 0.16	0.00 – 0.17
C12-OH	3-hydroxydodecanoyl-	0.00 – 0.02	0.00 – 0.02
C14	Tetradecanoyl-	0.00 – 0.07	0.00 – 0.05
C14:1	Tetradecenoyl-	0.00 – 0.23	0.00 – 0.16
C14:2	Tetradecadienoyl-	0.00 – 0.12	0.00 – 0.12
C14:OH	3-hydroxytetradecanoyl-	0.00 – 0.02	0.00 – 0.02
C14:1-OH	3-hydroxytetradecenoyl-	0.00 – 0.03	0.00 – 0.02
C16	Palmitoyl-	0.00 – 0.10	0.00 – 0.10
C16-DC*†	Dicarboxypalmitoyl-	--	--
C16:1	Palmitoleyl-	0.00 – 0.05	0.00 – 0.04
C16-OH	3-hydroxypalmitoyl-	0.00 – 0.01	0.00 – 0.01
C16:1-OH	3-hydroxypalmitoleyl-	0.00 – 0.01	0.00 – 0.01
C18	Stearoyl-	0.00 – 0.05	0.00 – 0.04
C18:1	Oleyl-	0.00 – 0.16	0.00 – 0.17
C18:1-DC*†	Dicarboxylic-	--	--
C18:2	Linoleyl-	0.00 – 0.08	0.00 – 0.10
C18-OH	3-hydroxystearoyl-	0.00 – 0.01	0.00 – 0.01

Table 3 (cont'd)

Acylcarnitine species	Chemical name (as -carnitine)	Reference range (plasma) (µmol/L)	
		8 days – 7 years	≥8 years
C18:1-OH	3-hydroxyoleyl-	0.00 – 0.01	0.00 – 0.01
C18:2-OH	3-hydroxylinoleyl-	0.00 – 0.01	0.00 – 0.01

*Species not currently reported by most laboratories

-- Species not currently reported by ARUP

(53,54). The AEDs carbamazepine, phenytoin, and phenobarbital are also linked to carnitine depletion (53). Other risk factors associated with carnitine deficiency include: age less than 10 years old, diagnosis of certain neurologic conditions, low intake of meat and dairy products (the primary sources of dietary carnitine), and past or present enteral or parenteral feeding (53).

Metabolic effects of carnitine deficiency during ketogenic diet treatment

For children with epilepsy who are treated with ketogenic diet therapy, especially those with a history of treatment with multiple AEDs, carnitine deficiency occurs more frequently than in the general population and results in metabolic consequences (37,56). When tissue carnitine concentration is low during initiation or maintenance of ketogenic diet treatment, fatty acids are not efficiently oxidized and ketone production is reduced. As a consequence, the limited amounts of circulating and cellular glucose (generated endogenously through gluconeogenesis or exogenously from small amounts of consumed dietary carbohydrate) are oxidized for energy without an adequate compensatory rise in ketones. This may result in hypoketotic hypoglycemia. Impaired ketone production and lower circulating concentration of ketones may also negatively affect seizure control, although this has yet to be determined.

Observations of carnitine deficiency during ketogenic diet treatment

In 2001, Berry-Kravis, et al., evaluated plasma total carnitine concentrations in 46 patients (age 1 – 24 years). Deficiency in this study was defined as total plasma carnitine concentration less than 31 μmol/L for males and less than 25 μmol/L for females (Metabolic Analysis Laboratories, Madison, WI). Of this population, 3 patients were found to be deficient in total carnitine at baseline prior to diet initiation and were supplemented with L-carnitine. Of the 43 initially unsupplemented patients following ketogenic diet initiation, 6 (18%) experienced a low carnitine concentration and required carnitine supplementation within the first 1 – 12 months of treatment. Average total carnitine of all patients who were never supplemented with carnitine was lower both 1 and 6 months after diet initiation, but increased at 12 and 24 months after initiation. The acyl/free carnitine concentration ratio was abnormal (>0.4) for all patients both supplemented and unsupplemented, ranging from 0.44 – 3.25 and found to be stable-to-decreasing from 1 month after ketogenic diet initiation onward (36). This was thought to be indicative of degree of ketosis rather than carnitine deficiency. No patients showed clinical symptoms of carnitine deficiency (defined as weakness, fatigue, changes in motor skills or cognition, decrease in tone, or signs of cardiac or liver dysfunction for the sake of this study).

In 2006, Coppola, et al., assessed plasma free carnitine concentrations prospectively in 11 patients who initiated the ketogenic diet in addition to continuing their current AED therapy. Carnitine deficiency was defined as free plasma carnitine concentration less than 20 μmol/L for this study. Participants had plasma free carnitine concentration assessed at baseline and after 3 and 12 months of ketogenic diet treatment. No participants experienced a plasma free carnitine concentration below 20

µmol/L during ketogenic diet treatment (53).

Carnitine supplementation during ketogenic diet treatment

Pharmacologic doses of carnitine during ketogenic diet treatment are typically administered at 10, 50, or 100 mg/kg for children who demonstrate or are at risk for secondary carnitine deficiency, with an initial recommended dose of 50 mg/kg/day and a maximum total daily dose of 3 g carnitine. The bioavailability of supplemental carnitine is thought to be 14 – 16% for doses of up to 100 mg/kg/day (31). Supplemental carnitine is generally well tolerated and toxic effects related to high-dose L-carnitine have not been reported. Carnitine supplementation may cause mild gastrointestinal symptoms, including: nausea, vomiting, abdominal cramps, gastritis, and diarrhea. Supplements providing more than 3 g/day of carnitine may cause a "fishy" body odor.

The current stance on carnitine supplementation for ketogenic diet patients varies widely between institutions. The consensus among experts in the field is that carnitine should only be supplemented when there is a true demonstrated biochemical need (indicated either by depleted lab values or a symptomatic deficiency) (12). However, many parents whose children are treated by the ketogenic diet choose to supplement carnitine either through prescriptions from their physician or from over-the-counter sources. This subjective use of supplemental carnitine has resulted in many anecdotal reports of “improved well-being, energy levels and seizure control,” especially if ketone levels have previously been depressed despite plasma carnitine concentration within the normal reference range (12).

The Oregon Health & Science University (Portland, OR) supplements carnitine in the form of L-carnitine (Carnitor®, Sigma Tau Pharmaceuticals, Gaithersburg, MD) when

medically indicated. L-carnitine is provided as a white, crystalline, hygroscopic powder in the form of tablets that contain 330 mg of L-carnitine each. Peak plasma concentration of L-carnitine occurs approximately 3 hours after a dosage of 50 mg/kg of Carnitor® (57). For a 20 kg child receiving 10, 50, or 100 mg/kg/day of L-carnitine, the yearly cost of treatment would be approximately $360, $1,800, and $3,600, respectively, at a cost of $1.60 per tablet.

Recent controversy regarding carnitine supplementation

A recent study linked L-carnitine supplementation to accelerated atherosclerosis, which is defined as the accumulation of arterial plaque. Atherosclerosis is a known risk factor for cardiovascular disease. Promotion of atherosclerosis secondary to supplemental carnitine administration is theorized to occur through microbial metabolism of ingested (but unabsorbed) L-carnitine to produce the metabolite trimethylamine-N-oxide (58). The theorized impact of L-carnitine supplementation and potential resultant trimethylamine-N-oxide formation on pediatric patients adherent to a ketogenic diet is minimal due to the restricted protein intake and therefore low consumption of dietary meat that is mandated by classic ketogenic diet therapy. Regardless, this possible harmful effect has placed the burden of proof on current research to demonstrate that the benefits of L-carnitine supplementation outweigh the potential risks.

CHAPTER 3

Specific Aims

The burden of proof rests on current research to demonstrate whether there is a true need for carnitine supplementation in patients treated with the ketogenic diet for intractable epilepsy. As a first step towards this outcome, the goal of this project was to determine the impact of the ketogenic diet on plasma carnitine concentration in this population, as well as to assess potential associations between plasma carnitine concentration and response to ketogenic diet therapy. To accomplish this goal, we completed a medical record review of pediatric patients with intractable epilepsy treated with the ketogenic diet who were not supplemented with carnitine.

The specific aims of this study were to:

1) Determine the impact of the ketogenic diet on plasma carnitine concentration in pediatric patients with intractable epilepsy by measuring plasma free carnitine, acylcarnitine, and total carnitine concentrations, acyl/free carnitine concentration ratio, and individual acylcarnitine species concentrations.

   Hypothesis: We hypothesized that mean plasma free and total carnitine concentrations would be significantly lower, mean plasma acylcarnitine concentration and acyl/free carnitine concentration ratio would be significantly higher, and mean plasma acylcarnitine species concentrations would reflect

ketosis (elevated C2 and C4-OH acylcarnitine species) at 1, 3, and 6 months after initiating the ketogenic diet.

2) Determine the association between degree of ketosis, measured by plasma β-hydroxybutyrate concentration, and carnitine, measured by plasma free carnitine, acylcarnitine, and total carnitine concentrations, and acyl/free carnitine concentration ratio in pediatric patients with intractable epilepsy treated with the ketogenic diet.

   Hypothesis: We hypothesized that plasma free carnitine, acylcarnitine, and total carnitine concentrations, and acyl/free carnitine concentration ratio would be positively correlated with plasma β-hydroxybutyrate concentration at 1, 3, and 6 months after initiating the ketogenic diet.

3) Determine the relationship of plasma free carnitine, acylcarnitine, and total carnitine concentrations and acyl/free carnitine concentration ratio with reduction in seizure frequency and/or number of AEDs required for pediatric patients with intractable epilepsy treated with the ketogenic diet.

   Hypothesis: We hypothesized that patients with higher plasma free carnitine, acylcarnitine, and total carnitine concentrations and acyl/free carnitine concentration ratio would be more likely to demonstrate a reduction in seizure frequency and number of AEDs required at 1, 3, and 6 months after initiating the ketogenic diet.

CHAPTER 4

Methods

Patient population

There are currently more than 75 patients actively enrolled in dietary treatment for intractable epilepsy and at least 60 patients treated with the ketogenic diet at the Doernbecher Children's Hospital Ketogenic Diet Clinic (Portland, OR). Doernbecher Children's Hospital is a nationally ranked children's hospital affiliated with the Oregon Health & Science University (OHSU) (Portland, OR). Typically 1 – 2 new patients are scheduled each month for inpatient admissions to initiate the ketogenic diet. Patients are asked to continue ketogenic diet therapy for a minimum of 3 months, although some patients discontinue the ketogenic diet before this point.

To be considered for ketogenic diet initiation for the treatment of intractable epilepsy at the Doernbecher Children's Hospital Ketogenic Diet Clinic, patients must have:

1) failed to achieve good seizure control with at least two AEDs,
2) been referred by a pediatric neurologist, and
3) been less than 18 years of age at the time of treatment initiation.

General design

We completed a medical record review to examine the impact of the ketogenic

diet on plasma carnitine concentration in children with intractable epilepsy, and to assess the potential associations between plasma carnitine concentration and response to ketogenic diet therapy. Screening and data collection were completed through electronic medical record audits (Epic Hyperspace 2012, Verona, WI). Medical records were reviewed retrospectively for children who initiated ketogenic diet treatment prior to February of 2014 and prospectively for children who initiated ketogenic diet treatment during or after February of 2014.

Screening and selection for medical record review

All patient medical records tracked by the Ketogenic Diet Clinic dietitians were selected for screening. Records were also identified by surveillance of the Ketogenic Diet Clinic schedule between November 2014 and March 2015. Patient medical records were included in this review if:

1) patients were prescribed a ketogenic diet ratio of at least 3 grams of fat for every 1 gram of combined carbohydrate and protein,
2) patients had sufficiently complete laboratory results (i.e., met minimum requirements of results available before ketogenic diet initiation and at one other time point),
3) patients were not supplemented with L-carnitine during ketogenic diet treatment, and
4) patients were less than 18 years of age.

Medical records of patients who were screened but not included in this study were characterized by reason for exclusion. Patient medical records were excluded from review if:

1) patients were prescribed a ketogenic diet ratio of less than 3 grams of fat for every 1 gram of combined carbohydrate and protein,
2) laboratory results were incomplete (i.e., minimum requirements of results available before ketogenic diet initiation and at one other time point not met),
3) patients were supplemented with L-carnitine during ketogenic diet treatment,
4) patients were older than 18 years of age, and
5) patients did not tolerate the ketogenic diet.

Although patients who received supplemental carnitine at baseline or before their second follow-up were excluded from the study, patients who had supplemental carnitine administered after their second follow-up had all data prior to the time of supplementation included and all data following the time of supplementation excluded.

Institutional review board approval, assent/consent, and privacy

This research protocol was submitted to and approved by the OHSU Institutional Review Board (IRB) prior to medical record review. Patients who attended standard clinical visits to the Ketogenic Diet Clinic between November 2014 and March 2015 were formally consented using an IRB-approved research consent and authorization form (Appendix A). Children provided written informed assent if able (Appendix B). Participants who did not come into a clinic visit during the determined consent window were included in this study under a Health Insurance Portability and Accountability Act (HIPPA) waiver.

All information abstracted from medical records remained strictly confidential. The only personnel with access to data were study investigators. All data associated

with patient identifiers were stored on secure, password-protected, OHSU network computers or in key-locked file cabinets. Cloud storage (OHSU Box) was used for secure information as appropriate. Any data stored in other locations was identified by randomly generated number identifiers only.

Doernbecher Children's Hospital ketogenic diet administration protocol

Ketogenic diet therapy was initiated in accordance with clinic protocol as medically appropriate. Ketogenic diet initiation occurred over a 3-day hospital admission. Standard protocol at the Ketogenic Diet Clinic does not include fasting or fluid restriction during ketogenic diet initiation. Each subject consumed a diet designed by the pediatric dietitians who staff the Ketogenic Diet Clinic at Doernbecher Children's Hospital. Ketogenic diet ratios were calculated using Ketocalculator (Beth Zupec-Kania and LifeTime Computing, Inc., Brown Deer, WI). Nutritional formulas prescribed for patients who required partial or complete formula feeding included: KetoCal 4:1 liquid (unflavored or vanilla), KetoCal 4:1 powder, KetoCal 3:1 powder (Nutricia, Gaithersburg, MD), and Ross Carbohydrate Free soy formula (Abbott Nutrition, Columbus, OH). Following discharge from the hospital, the diet was prepared and served by the parents of the patients as instructed by the pediatric dietitian.

Doernbecher Children's Hospital supplemental carnitine protocol

Current treatment protocol is to supplement with L-carnitine (Carnitor, Sigma-Tau Pharmaceuticals, Gaithersburg, MD) only if the patient has a demonstrated biochemical need. Occasional exceptions are made if a patient has a history of treatment with valproic acid. When supplementing with L-carnitine, 50 – 100 mg/kg/day

is given in divided doses up to a maximum dose of 3 g/day. Few children currently receiving ketogenic dietary treatment at the Ketogenic Diet Clinic are supplemented with L-carnitine at this time.

Doernbecher Children's Hospital biochemical assessment protocol

Plasma samples were analyzed for free carnitine, acylcarnitine, and total carnitine concentrations by tandem mass spectroscopy. Acyl/free carnitine concentration ratio was calculated and reported by the laboratory. Plasma concentrations of individual acylcarnitine species were quantified by tandem mass spectrometry. Plasma β-hydroxybutyrate concentration was assessed by quantitative enzymatic analysis. Carnitine analyses were performed by ARUP Laboratories (Salt Lake City, UT), Quest Diagnostics (Madison, NJ), and Duke Children's Hospital & Health Center (Durham, NC). Plasma β-hydroxybutyrate analyses were performed at the previously mentioned laboratories as well as at OHSU Knight Diagnostic Laboratories (Portland, OR).

Patients who were treated at the Ketogenic Diet Clinic during or after February of 2014 were prescribed the following biochemical analysis protocol:

<u>Baseline (prior to initiation) and 6 months after ketogenic diet initiation:</u>

- Plasma free carnitine, acylcarnitine, and total carnitine concentrations and acyl/free carnitine concentration ratio
- Plasma acylcarnitine species concentrations
- Plasma β-hydroxybutyrate concentration

<u>1 and 3 months after ketogenic diet initiation:</u>

- Plasma free carnitine, acylcarnitine, and total carnitine concentrations and

acyl/free carnitine concentration ratio

- Plasma β-hydroxybutyrate concentration

See Appendix C for a full summary of current laboratory analysis protocols. Patients who received ketogenic diet treatment at the Ketogenic Diet Clinic before February of 2014 had biochemical analyses performed according to the clinical protocols followed and preference of the supervising physician who managed care at time of treatment.

Statistical analysis

Data regarding participant characteristics, including demographics, medical diagnoses, ketogenic diet ratio, AED regimen, and treatment measures (seizure reduction and reduction in number of AEDs) were summarized. Participants were categorized according to carnitine status ("below", "within", or "above" normal reference ranges) according to plasma concentrations of free carnitine, acylcarnitine, and total carnitine and acyl/free carnitine concentration ratio.

Means and standard deviations of plasma free carnitine, acylcarnitine, and total carnitine, acyl/free carnitine concentration ratio, and acylcarnitine species concentrations were calculated at baseline and 1, 3, and 5 – 8 months after initiating ketogenic diet therapy. These time points are referred to from this point onward as assessment windows. Participants with more than one laboratory result within a single assessment window had results averaged for computation of means. Difference in means available for multiple assessment windows was assessed by generalized estimating equations (GEE) method (59). GEE was selected because it is designed specifically to analyze longitudinal data while controlling for individuals who contribute

data during multiple assessment windows. GEE compares the mean of a variable at each assessment window to the mean of the variable at baseline only, which also allows for more sensitive detection of significant differences between means assessed over time. A paired t-test was employed to determine differences in means for variables available at only two assessment windows.

Mean percent change in concentrations between assessment windows was defined as the mean of observed percent changes between baseline and observed concentration [[(observed-baseline)/baseline]x100] for each individual participant during an assessment window.

Linear regression analysis was performed for plasma carnitine and β-hydroxybutyrate concentrations from 1 – 8 months of ketogenic diet treatment. A correlation coefficient was computed. Standard errors estimated by the model were adjusted to account for subjects who contributed multiple measurements within an assessment window and during the treatment course.

An odds ratio and 95% confidence interval were calculated using logistic regression to compare the effect of plasma carnitine status and plasma carnitine concentration (below the 25th percentile for all abstracted concentrations compared to at least the 25th percentile for all abstracted concentrations) on achievement of: 50% or greater reduction in seizure frequency from 1 – 8 months of ketogenic diet treatment, and reduction in number of AEDs prescribed from 3 – 8 months of ketogenic diet treatment. Similar to regression models described above, standard errors used for estimation and testing of odds ratios were adjusted to account for subjects who

contributed multiple measurements within an assessment window and during the treatment course.

Microsoft Excel (version 14.4.8, Redmond, WA), StataCorp LP STATA/IC (version 13.1, College Station, Texas), and Graphpad Prism (version 6.0, La Jolla, CA) were used for data analysis. Statistical output was considered significant at $p<0.05$.

CHAPTER 5

Results

We completed a prospective and retrospective medical record review of pediatric patients with intractable epilepsy who were treated with the ketogenic diet at Doernbecher Children's Hospital to determine:

1) the impact of the ketogenic diet on plasma carnitine concentration,
2) possible correlations between plasma carnitine and β-hydroxybutyrate concentration, and
3) the association between plasma carnitine concentration and reduction in seizure frequency and number of AEDs prescribed.

Screening and selection process

The medical records of 109 patients were reviewed using electronic health record software. Patient medical records were excluded from review if: patients were older than 18 years of age (n=3); patients were prescribed a modified Atkins diet (n=10); patients were prescribed a ketogenic diet ratio of less than 3 grams of fat for every 1 gram of combined carbohydrate and protein (n=29); patients did not tolerate the ketogenic diet (n=1); patients were supplemented with carnitine during ketogenic diet treatment (n=11); or laboratory results were incomplete (i.e., minimum requirements of results available before ketogenic diet initiation and at one other time point not met)

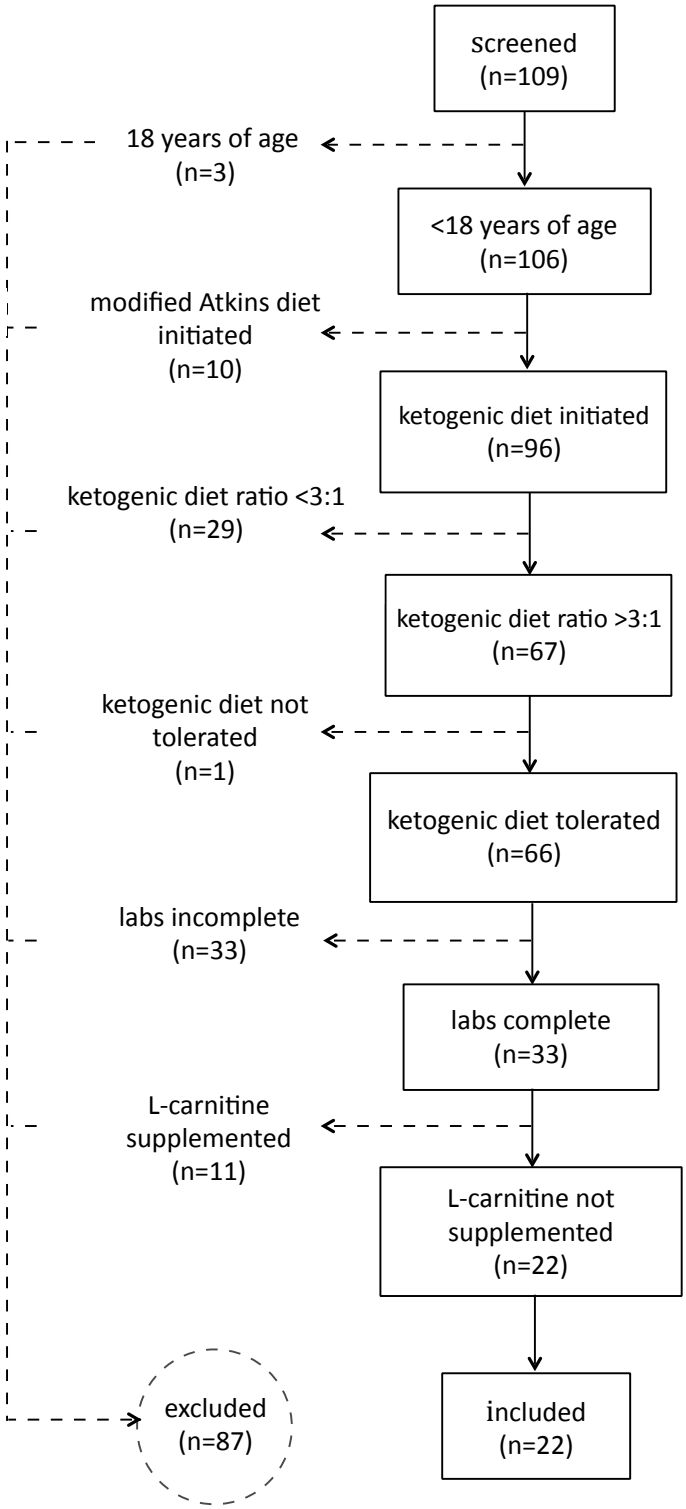


Figure 1: Results of participant selection process

(n=33). Of the 109 patient medical records that were screened, 87 patient medical records (80%) were excluded from this study and 22 patient medical records (20%) were included in this study. Results of the selection process for this medical record review are illustrated in Figure 1.

Participant characteristics

Participant characteristics (n=22) at time of ketogenic diet initiation are summarized in Table 4. Participants included both male (n=11, 50%) and female (n=11, 50%) children. The mean age of participants was 3.9 ± 4.8 years, with a range of 0.08 – 16.0 years. Median age of participants was 1.3 years, and the majority was no older than 3 years of age (n=14, 64%). The dietitian-established ketogenic diet goals for participants included ratios of 3:1 (n=10, 45%), 3.5:1 (n=2, 9%), and 4:1 (n=10, 45%) at initiation. Ketogenic diets were provided entirely as nutritional formula with or without additional modular nutritional products for the majority of our participants (n=17, 77%), either as KetoCal nutritional formula (n=13) or as Ross Carbohydrate Free soy formula (n=4).

AED polytherapy (i.e., more than one AED prescribed) at ketogenic diet initiation was by far most common (n=15, 68%), followed by monotherapy (n=3, 14%), and no drug therapy (n=4, 18%). The most frequently prescribed AEDs included phenobarbital (n=8, 36%), levetiracetam (n=7, 32%), topiramate (n=5, 23%), and zonisamide (n=5, 23%). Study participants experienced a wide variety of epilepsy etiologies that can be predominantly grouped as brain injury, genetic abnormality, or unknown cause. Participants also demonstrated several different epilepsy syndromes, including Lennox-

Table 4: Participant characteristics at ketogenic diet initiation (n=22)

Category	Number of participants	Category	Number of participants
Sex		Epilepsy etiology	
Male	11 (50%)	Hypoxic ischemic encephalopathy	3 (14%)
Female	11 (50%)	Periventricular leukomalacia	2 (9%)
Age (years)		Encephalitis	1 (5%)
Mean ± SD	3.9 ± 4.8	Congenital cytomegalovirus infection	1 (5%)
Median	1.33		
Range	0.08 – 16.0	Cyclin-dependent kinase-like 5 mutation	1 (5%)
Number of participants by age			
<1 year	7 (32%)	Cerebral palsy	1 (5%)
1 – 3 years	7 (32%)	Type II voltage-gated sodium channel mutation	1 (5%)
4 – 10 years	5 (23%)		
>10 years	3 (14%)	Methyl CpG binding protein 2 duplication syndrome	1 (5%)
Ketogenic diet ratio (g) fat:PRO+CHO			
3:1	10 (45%)	Pyruvate dehydrogenase deficiency syndrome	1 (5%)
3.5:1	2 (9%)		
4:1	10 (45%)	Syntaxin binding protein 1 mutation	1 (5%)
Ketogenic diet source			
Nutritional formula	17 (77%)	Glucose transporter 1 deficiency syndrome	1 (5%)
Foods	3 (14%)		
Nutritional formula + foods	2 (9%)	Koolen-de Vries syndrome (17q21.31 microdeletion)	1 (5%)
Number of antiepileptic drugs			
0	4 (18%)	Trisomy 13 syndrome	1 (5%)
1	3 (14%)	Aicardi syndrome	1 (5%)
2	7 (32%)	Unknown	5 (23%)
3	5 (23%)	Epilepsy syndrome	
4	3 (14%)	Lennox-Gastaut syndrome	5 (23%)
Antiepileptic drugs prescribed*		West syndrome (infantile spasms)	3 (14%)
Phenobarbital	8 (36%)	Ohtahara syndrome (early infantile epileptic encephalopathy)	2 (9%)
Levetiracetam	7 (32%)		
Topiramate	5 (23%)	Early myoclonic encephalopathy	1 (5%)
Zonisamide	5 (23%)	Nonspecific (focal, multifocal or other generalized seizures)	11 (50%
Clonazepam	3 (14%)		
Lamotrigine	3 (14%)		
Vigabatrim	3 (14%)		
Valproic acid	2 (9%)		
Clorazepate	2 (9%)		

Table 4 (cont'd)

Category	Number of participants
Antiepileptic drugs prescribed (cont'd)*	
Rufinamide	2 (9%)
Oxcarbazepine	1 (5%)
Clobazam	1 (4%)
Phenytoin	1 (4%)
Methsuximide	1 (5%)

*As percentage of total number of participants (n=22)

Gastaut syndrome (n=5, 23%) and West syndrome (n=3, 14%). Half of the patients experienced a nonspecific epilepsy syndrome characterized by focal, multifocal or other generalized seizures (n=11, 50%).

Treatment measures

Participants included in this study initiated ketogenic diet therapy between

Table 5: Treatment measures at most recent follow-up (n=22)

Category	Number of participants
On ketogenic diet therapy at time of review	14 (64%)
Ketogenic diet therapy discontinued	8 (36%)
Duration of ketogenic diet therapy if discontinued	
<3 months*	1 (13%)
6 – 12 months	2 (25%)
>12 months	5 (63%)
Change in seizure frequency	
No change or worse	4 (18%)
<50% reduction	6 (27%)
50 – 99% reduction	4 (18%)
Seizure free	8 (36%)
Change in number of antiepileptic drugs	
No change or increased	12 (55%)
Reduced by at least one	10 (45%)

*As percentage of those who discontinued ketogenic diet therapy (n=8)

January 2010 and November 2014. Treatment measures observed at each participant's most recent follow-up visit are summarized in Table 5. The majority of participants (n=14, 64%) continued to be treated with the ketogenic diet at their most recently recorded clinic visit. Of the participants who discontinued ketogenic diet treatment (n=8), the majority was treated for more than 12 months (n=5, 63%). The remaining participants were treated with ketogenic diet for fewer than 3 months (n=1, 13%) or for 6 – 12 months (n=2, 25%).

More than half of participants had at least a 50% reduction in seizure frequency (n=12, 55%), of whom two-thirds experienced complete seizure freedom (n=8). About half of participants were able to reduce the number of AEDs they were prescribed by one or more medications during ketogenic diet treatment (n=10, 45%). Of those 12 participants who experienced at least a 50% reduction in seizure frequency, more than half were also able to reduce the number of AEDs they required by at least one medication (n=7, 58%).

Biochemical assessment of participants

Assessment windows were defined as: baseline prior to ketogenic diet initiation, 1 month after ketogenic diet initiation, 3 months after ketogenic diet initiation, and 5 – 8 months after ketogenic diet initiation. Timing and type of biochemical analyses for each of the 22 individual participants varied according to: clinical protocols followed, the supervising physician who managed care at time of treatment, and patient adherence to the recommended follow-up schedule.

As shown in Table 6, plasma free carnitine, acylcarnitine, and total carnitine

Table 6: Available data for biochemical assessment of carnitine

Participant ID	Plasma free carnitine, acylcarnitine, and total carnitine concentrations and acyl/free carnitine concentration ratio				Plasma acylcarnitine species concentrations	
(n=22)	Baseline (n=10)	1 mo. (n=6)	3 mo. (n=6)	5 – 8 mo. (n=7)	Baseline (n=17)	5 – 8 mo. (n=17)
6727	•*	•	•	•	•	•
6346	•	•	•	•	•	•
8988	•	•	•	•		
7292	•	•	•			
5559	•	•	•			
6949	•	•				
1722	•		•	•	•	•
2932	•			•		
4229	•			•	•	•
4700	•			•	•	•
1233					•	•
5051					•	•
3909					•	•
4981					•	•
7574					•	•
3602					•	•
3040					•	•
8113					•	•
8098					•	•
7284					•	•
6471					•	•
1986					•	•

*• = data available

concentrations and the corresponding acyl/free carnitine concentration ratio were assessed for certain participants prior to ketogenic diet initiation (n=10), 1 month after ketogenic diet initiation (n=6), 3 months after ketogenic diet initiation (n=6), and 5 – 8 months after ketogenic diet initiation (n=7). Only 3 participants had full adherence to the biochemical analysis protocol for plasma free carnitine, acylcarnitine, and total carnitine concentrations and acyl/free carnitine concentration ratio from baseline through 5 – 8 months of treatment without any missing data. Five participants had full adherence to the biochemical analysis protocol for plasma free carnitine, acylcarnitine,

Table 7: Available data for biochemical assessment of acylcarnitine species

Participant ID	Baseline								5 – 8 months							
	C2 – C4	C4-OH	C8 – C10:1	C10:2	C12 – C16	C16:1	C18	C18:1 – C18:2	C2 – C4	C4-OH	C8 – C10:1	C10:2	C12 – C16	C16:1	C18	C18:1 – C18:2
(n=17)	(n=17)	(n=9)	(n=17)	(n=9)	(n=17)	(n=9)	(n=9)	(n=17)	(n=17)	(n=7)	(n=17)	(n=4)	(n=17)	(n=13)	(n=13)	(n=17)
3602	•*	•	•	•	•	•		•	•	•	•	•	•			•
8098	•	•	•	•	•			•	•	•	•	•	•			•
8113	•	•	•	•	•			•	•	•	•	•	•			•
3040	•	•	•	•	•			•	•	•	•	•	•			•
5051	•	•	•	•	•			•	•	•	•		•	•	•	•
1986	•	•	•	•	•			•	•		•		•	•	•	•
6471	•	•	•	•	•			•	•		•		•	•	•	•
7284	•	•	•	•	•			•	•		•		•	•	•	•
7574	•	•	•	•	•			•	•		•		•	•	•	•
4229	•		•		•	•	•	•	•	•	•		•	•	•	•
4700	•		•		•	•	•	•	•	•	•		•	•	•	•
1233	•		•		•	•	•	•	•		•		•	•	•	•
1722	•		•		•	•	•	•	•		•		•	•	•	•
3909	•		•		•	•	•	•	•		•		•	•	•	•
4981	•		•		•	•	•	•	•		•		•	•	•	•
6346	•		•		•	•	•	•	•		•		•	•	•	•
6727	•		•		•	•	•	•	•		•		•	•	•	•

*• = data available

and total carnitine concentrations and acyl/free carnitine concentration ratio from baseline through 3 months of treatment without any missing data.

As shown in Table 6, acylcarnitine species concentrations were assessed for 17 participants prior to ketogenic diet initiation and again 5 – 8 months after ketogenic diet initiation (n=17). As shown in Table 7, the specific acylcarnitine species reported varied for individual participants. Some biochemical analyses reported certain additional acylcarnitine species (e.g., C4-OH and C10:2) and some did not report certain acylcarnitine species (e.g., C16:1 and C18).

Impact of the ketogenic diet on carnitine concentration

The first aim of this research was to determine the impact of the ketogenic diet on plasma carnitine concentration in pediatric participants with intractable epilepsy. Mean plasma free carnitine, acylcarnitine, and total carnitine concentrations and the acyl/free carnitine concentration ratio are summarized before and after initiation of ketogenic diet treatment in Table 8 and Figures 2a – c. Summary data is provided for participants with complete biochemical analysis at baseline, 1 month, 3 months, and 5 – 8 months after ketogenic diet initiation (n=3, Figure 2a), and for those participants with complete biochemical analysis at baseline, 1 month, and 3 months after ketogenic diet initiation (n=5, Figure 2b), as well as for participants with data at baseline and at least one other time point (n=10, Figure 2c). Similar trends in plasma free carnitine, acylcarnitine, and total carnitine concentrations and acyl/free carnitine concentration ratio over time were observed regardless of the cohort analyzed. Mean plasma free carnitine concentration was significantly lower than baseline at 1 month for the longitudinal cohort of 5 participants ($p<0.05$). Mean plasma free carnitine concentration

Table 8: Mean plasma carnitine and acylcarnitine species concentrations before and after initiation of ketogenic diet treatment

Parameter	Plasma concentration (Mean ± SD)			
	Baseline	**1 month**	**3 months**	**5 – 8 months**
Longitudinal	**(n=3)**	**(n=3)**	**(n=3)**	**(n=3)**
Free carnitine (µmol/L)	33.8 ± 2.3	22.0 ± 11.3	16.7 ± 11.7*	20.3 ± 7.51*
Total carnitine (µmol/L)	44.3 ± 8.14	50.6 ± 11.0	46.3 ± 24.2	64.5 ± 13.9
Acylcarnitine (µmol/L)	10.5 ± 6.14	28.6 ± 6.37**	29.6 ± 12.5	44.2 ± 7.08**
Acyl/free carnitine ratio	0.30 ± 0.17	1.52 ± 0.78**	2.03 ± 0.55**	2.40 ± 0.62**
Longitudinal	**(n=5)**	**(n=5)**	**(n=5)**	
Free carnitine (µmol/L)	34.9 ± 3.31	24.38 ± 9.63*	22.4 ± 14.2*	
Total carnitine (µmol/L)	46.0 ± 6.67	56.16 ± 11.8	57.8 ± 24.5	
Acylcarnitine (µmol/L)	11.1 ± 6.64	31.8 ± 6.24**	35.4 ± 11.8**	
Acyl/free carnitine ratio	0.32 ± 0.21	1.45 ± 0.60**	1.9 ± 0.66**	
Cross-sectional	**(n=10)**	**(n=6)**	**(n=6)**	**(n=7)†**
Free carnitine (µmol/L)	30.7 ± 11.2	23.5 ± 8.89	21.3 ± 13.0	22.7 ± 7.59
Total carnitine (µmol/L)	41.1 ± 11.1	54.5 ± 11.4*	57.0 ± 22.0	61.6 ± 12.5*
Acylcarnitine (µmol/L)	10.4 ± 5.3	31.0 ± 5.9**	35.7 ± 10.6**	38.8 ± 9.4**
Acyl/free carnitine ratio	0.47 ± 0.70	1.44 ± 0.53**	1.97 ± 0.61**	1.92 ± 0.79**
	(n=17)			**(n=17)†**
C2 (µmol/L)	7.33 ± 4.39			34.4 ± 13.9**
C3 (µmol/L)	0.37 ± 0.21			0.27 ± 0.12
C4 (µmol/L)	0.17 ± 0.07			0.19 ± 0.09
C8 (µmol/L)	0.10 ± 0.04			0.16 ± 0.07**
C8:1 (µmol/L)	0.21 ± 0.16			0.27 ± 0.11
C10 (µmol/L)	0.14 ± 0.06			0.29 ± 0.23*
C10:1 (µmol/L)	0.12 ± 0.08			0.30 ± 0.33*
C12 (µmol/L)	0.09 ± 0.09			0.08 ± 0.03
C12:1 (µmol/L)	0.08 ± 0.09			0.09 ± 0.04
C14 (µmol/L)	0.04 ± 0.05			0.05 ± 0.03
C14:1 (µmol/L)	0.10 ± 0.08			0.30 ± 0.35*
C14:2 (µmol/L)	0.06 ± 0.08			0.12 ± 0.12
C16 (µmol/L)	0.10 ± 0.10			0.09 ± 0.07
C18:1 (µmol/L)	0.13 ± 0.08			0.37 ± 0.18**
C18:2 (µmol/L)	0.10 ± 0.10			0.15 ± 0.07*
	(n=8)			**(n=8)†**
C16:1 (µmol/L)	0.03 ± 0.04			0.03 ± 0.02

Table 8 (cont'd)

Parameter	Plasma concentration (mean ± SD)			
	Baseline	**1 month**	**3 months**	**5 – 8 months**
	(n=8)			**(n=8)†**
C18 (µmol/L)	0.04 ± 0.02			0.07 ± 0.01*
	(n=5)			**(n=5)†**
C4-OH (µmol/L)	0.07 ± 0.12			0.49 ± 0.24*
	(n=4)			**(n=4)†**
C10:2 (µmol/L)	0.02 ± 0.02			0.02 ± 0.02

Different from baseline *p<0.05; **p<0.01

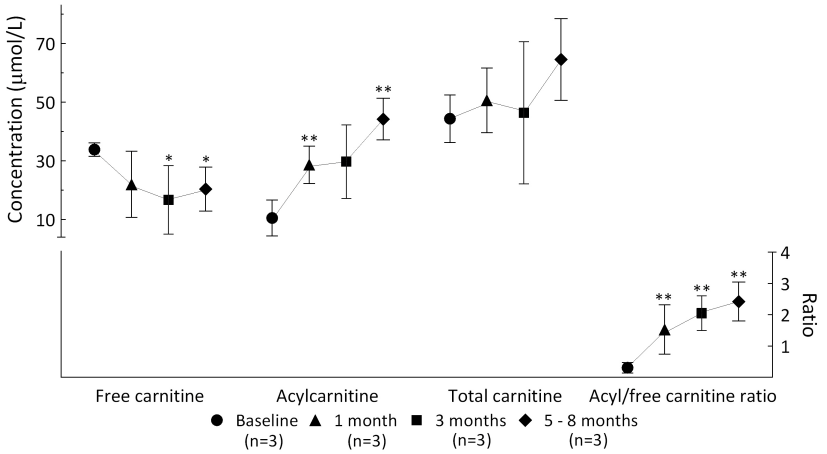
†Participants with more than one result had concentrations represented as a single average

was also significantly lower than baseline at 3 months for both cohorts of longitudinal participants (p=0.03 for both n=3 and n=5), and at 5 – 8 months for the longitudinal cohort of 3 participants (p=0.01).

Mean plasma acylcarnitine concentration was significantly higher than baseline at 1 month for both cohorts of longitudinal participants and for the larger cross-sectional cohort of participants (p<0.01 for all cohorts). Mean plasma acylcarnitine concentration was significantly higher than baseline at 3 months for the longitudinal cohort of 5 participants and the cross-sectional cohort of participants (p<0.01 for both cohorts). Mean plasma acylcarnitine concentration was significantly higher than baseline at 5 – 8 months for the longitudinal cohort of 3 participants and for the larger cross-sectional cohort of participants (p<0.01 for both cohorts).

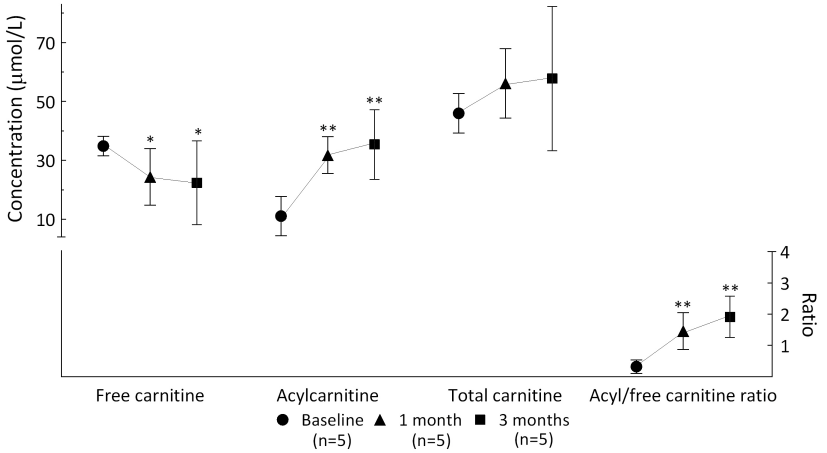
Mean plasma total carnitine concentration was significantly higher than baseline at 1 month and 5 – 8 months for only the cross-sectional cohort of participants (p=0.02 and p<0.01, respectively). Mean plasma acyl/free carnitine concentration ratio was significantly higher than baseline at 1 month and 3 months for both cohorts of

Figure 2a: Longitudinal analysis of mean plasma carnitine concentrations before and 1, 3, and 5 - 8 months after initiation of ketogenic diet treatment (n=3)



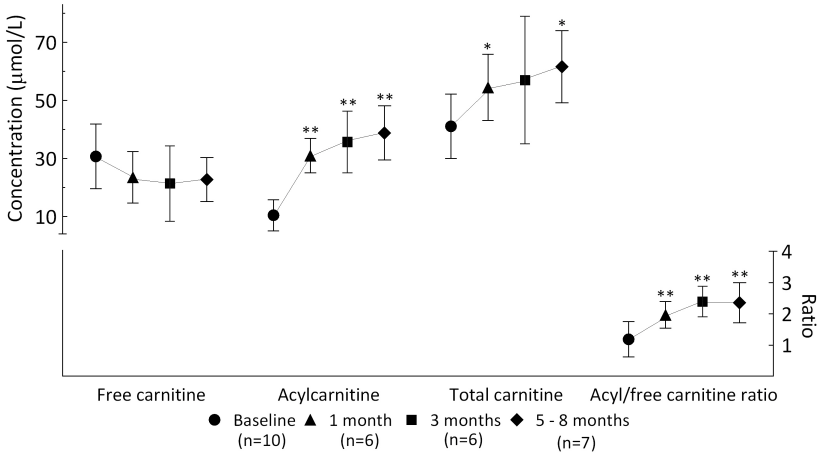
Mean ± SD; different from baseline * p<0.05; ** p<0.01

Figure 2b: Longitudinal analysis of mean plasma carnitine concentrations before and 1 and 3 months after initiation of ketogenic diet treatment (n=5)



Mean ± SD; different from baseline * p<0.05; ** p<0.01

Figure 2c: Cross-sectional analysis of mean plasma carnitine concentrations before and 1, 3, and 5 - 8 months after initiation of ketogenic diet treatment (n=10)

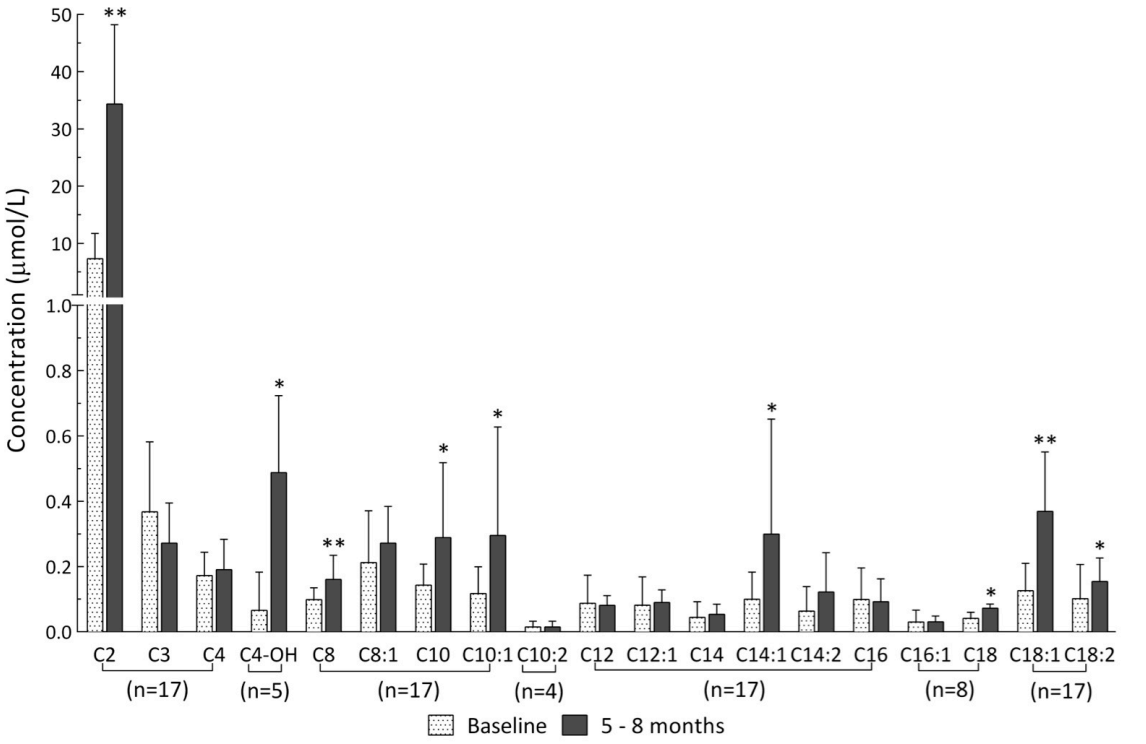


Mean ± SD; different from baseline * p<0.05; ** p<0.01

longitudinal participants and for the larger cross-sectional cohort of participants ($p<0.01$ for all cohorts). Mean plasma acyl/free carnitine concentration ratio was significantly higher than baseline at 5 – 8 months for the longitudinal cohort of 3 participants and for the cross-sectional cohort of participants ($p<0.01$ for both cohorts).

Mean acylcarnitine species concentrations, represented in Table 8 and Figure 3, were calculated for short-chain acylcarnitine species (C2 – C4 and C4-OH), saturated and unsaturated medium-chain acylcarnitine species (C8 – C12:1), and saturated and unsaturated long-chain acylcarnitine species (C12 – C18:2) for 17 participants at baseline and again 5 – 8 months after initiating ketogenic diet therapy. Participants who had a specific species reported at the first time point but not at the latter time point had

Figure 3: Longitudinal analysis of mean plasma acylcarnitine species concentrations before and 5 - 8 months after initiation of ketogenic diet treatment (n=17)



Mean ± SD; different from baseline * $p<0.05$; ** $p<0.01$

that species omitted from the analysis, but retained all other species for which concentrations were available at both time points.

Mean plasma concentrations were significantly higher than baseline at 5 – 8 months for the acylcarnitine species: C2, C4-OH, C8, C10, C10:1, C14:1, C18, C18:1, and C18:2. As shown in Table 9, the highest mean percent increases from baseline concentrations (calculated as the mean of all individual percent changes for an acylcarnitine species concentration) were observed in the acylcarnitine species C4-OH with a 711 ± 499% mean increase above baseline concentration (n=5), and C2 with a 488 ± 352% mean increase above baseline concentration (n=17). Significant increases from baseline concentration was also observed in C14:1 with a 300 ± 279% mean increase above baseline concentration (n=17), and C18:1 with a 290 ± 335% mean increase above baseline concentration (n=17). Less extreme increases that still exceeded a one-hundred mean percent increase from baseline concentration were observed in C18:2 with a 184 ± 247% mean increase above baseline concentration (n=17), C10:1 with a 180 ± 185% mean increase above baseline concentration (n=17), C14:2 with a 157 ± 166% mean increase above baseline concentration (n=17), C10 with a 130 ± 181% mean increase above baseline concentration (n=17), and C18 with a 112 ± 96% mean increase above baseline concentration (n=8).

Changes in carnitine status during ketogenic diet treatment

The number of participants with plasma carnitine concentrations categorized as "above", "within", or "below" the normal reference range during each assessment window is reported in Table 10. Plasma free and total carnitine concentrations were exclusively within or below normal reference range at baseline and after ketogenic diet

Table 9: Mean percent change from baseline of plasma carnitine and acylcarnitine species concentrations before and after initiation of ketogenic diet treatment

Parameter	Percent change in plasma concentration (mean ± SD)		
	1 month	3 months	5 – 8 months
Longitudinal	**(n=3)**	**(n=3)**	**(n=3)**
Free carnitine (%)	- 17 ± 31	- 30 ± 39	- 39 ± 26
Total carnitine (%)	10 ± 33	9 ± 60	53 ± 60
Acylcarnitine (%)	147 ± 253	256 ± 505	562 ± 618
Acyl/free carnitine ratio (%)	217 ± 250	443 ± 519	874 ± 495
Longitudinal	**(n=5)**	**(n=5)**	
Free carnitine (%)	- 24 ± 32	- 37 ± 37	
Total carnitine (%)	21 ± 33	32 ± 68	
Acylcarnitine (%)	266 ± 289	436 ± 499	
Acyl/free carnitine ratio (%)	481 ± 541	683 ± 396	
Cross-sectional	**(n=6)**	**(n=6)**	**(n=7)**
Free carnitine (%)	- 32 ± 31	- 37 ± 37	- 39 ± 26
Total carnitine (%)	21 ± 33	32 ± 68	53 ± 60
Acylcarnitine (%)	312 ± 259	436 ± 499	562 ± 618
Acyl/free carnitine ratio (%)	594 ± 489	683 ± 396	874 ± 495
			(n=17)*
C2 (%)			488 ± 352
C3 (%)			- 4 ± 69
C4 (%)			16 ± 48
C8 (%)			90 ± 139
C8:1 (%)			67 ± 99
C10 (%)			130 ± 181
C10:1 (%)			180 ± 185
C12 (%)			42 ± 99
C12:1 (%)			87 ± 170
C14 (%)			54 ± 86
C14:1 (%)			300 ± 279
C14:2 (%)			157 ± 166
C16 (%)			12 ± 70
C18:1 (%)			290 ± 335
C18:2 (%)			184 ± 247
			(n=8)
C16:1 (%)			95 ± 166
C18 (%)			112 ± 96

Table 9 (cont'd)			
Parameter	**Percent change in plasma concentration (mean ± SD)**		
	1 month	**3 months**	**5 – 8 months**
			(n=5)
C4-OH (%)			711 ± 499
			(n=4)
C10:2 (%)			- 20 ± 92

*Participants with more than one result had concentrations represented as a single average

initiation. Plasma acylcarnitine concentrations were exclusively within the normal reference range at baseline and at 1 month after ketogenic diet initiation, and were equally within or above the normal reference range at 3 and 5 – 8 months after ketogenic diet initiation. Plasma acyl/free carnitine concentration ratio was exclusively within or above the normal reference range before and after ketogenic diet initiation.

Individual and mean plasma free carnitine concentrations before and after initiation of ketogenic diet treatment are depicted in Figure 4. Participants who initiated ketogenic diet therapy within the normal reference range for plasma free carnitine concentrations demonstrated a relatively equal tendency to either remain within or drop below the reference range during treatment. Five participants with plasma free carnitine concentrations within the normal reference range at baseline dropped below the reference range between 1 and 3 months after initiation of ketogenic diet treatment.

Individual and mean plasma acylcarnitine concentrations before and after initiation of ketogenic diet treatment are depicted in Figure 5. All participants were within the normal reference range at baseline, and none crossed below the normal

reference range during the first 8 months of treatment. Six participants had plasma acylcarnitine concentrations above the normal reference range between 3 and 8 months and all 6 subsequently remained above the normal reference range throughout the first 8 months of treatment.

Individual and mean plasma total carnitine concentrations before and after initiation of ketogenic diet treatment are outlined in Figure 6. The majority of participants who initiated ketogenic diet treatment with plasma total carnitine concentrations within the normal reference range continued to maintain total carnitine concentrations within the normal reference range throughout the first 8 months of

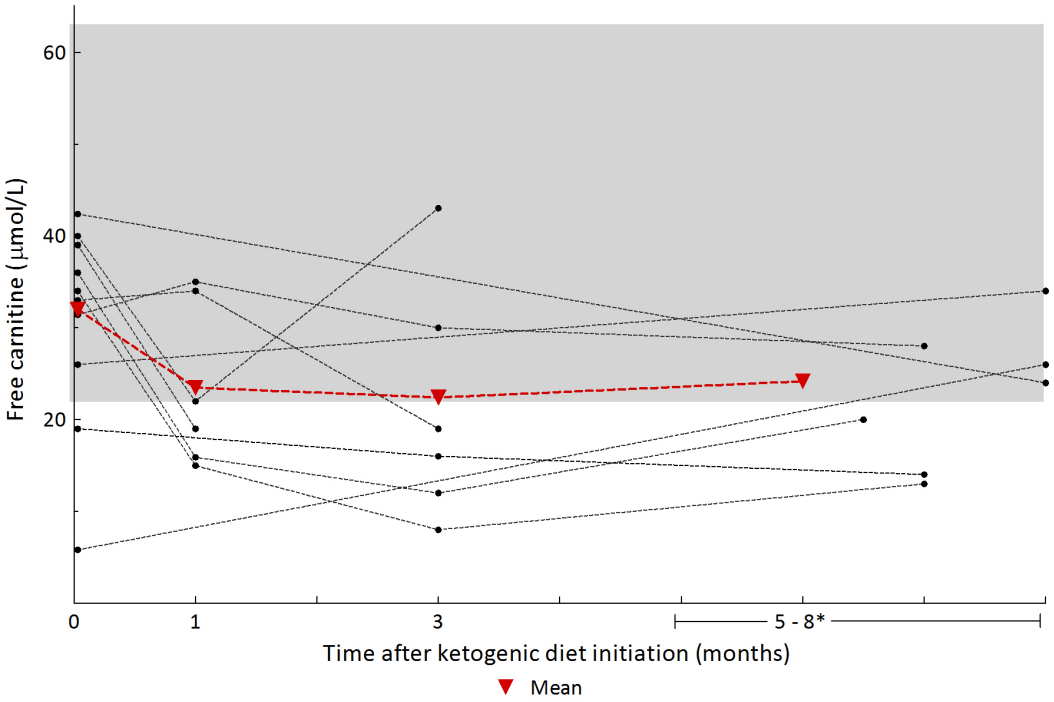
Table 10: Number of participants with plasma carnitine concentrations above, within, or below normal reference ranges before and after initiation of ketogenic diet treatment

Parameter	Reference range		Number of participants in each category			
			Baseline (n=10)	1 month (n=6)	3 months (n=6)	5 – 8 months (n=7)†
Free carnitine (µmol/L)	High	>63	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	WNL*	22 – 63	8 (80%)	3 (50%)	2 (33%)	4 (57%)
	Low	<22	2 (20%)	3 (30%)	4 (67%)	3 (43%)
Acylcarnitine (µmol/L)	High	>38	0 (0%)	0 (0%)	3 (50%)	3 (43%)
	WNL	3 – 38	10 (100%)	6 (100%)	3 (50%)	4 (57%)
	Low	<3	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total carnitine (µmol/L)	High	>90	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	WNL	31 – 90	8 (80%)	6 (100%)	5 (83%)	7 (100%)
	Low	<31	2 (20%)	0 (0%)	1 (17%)	0 (0%)
Acyl/free carnitine ratio	High	>0.9	1 (10%)	5 (83%)	6 (100%)	6 (86%)
	WNL	0.1 – 0.9	9 (90%)	1 (17%)	0 (0%)	1 (14%)
	Low	<0.1	0 (0%)	0 (0%)	0 (0%)	0 (0%)

*WNL, within normal limits

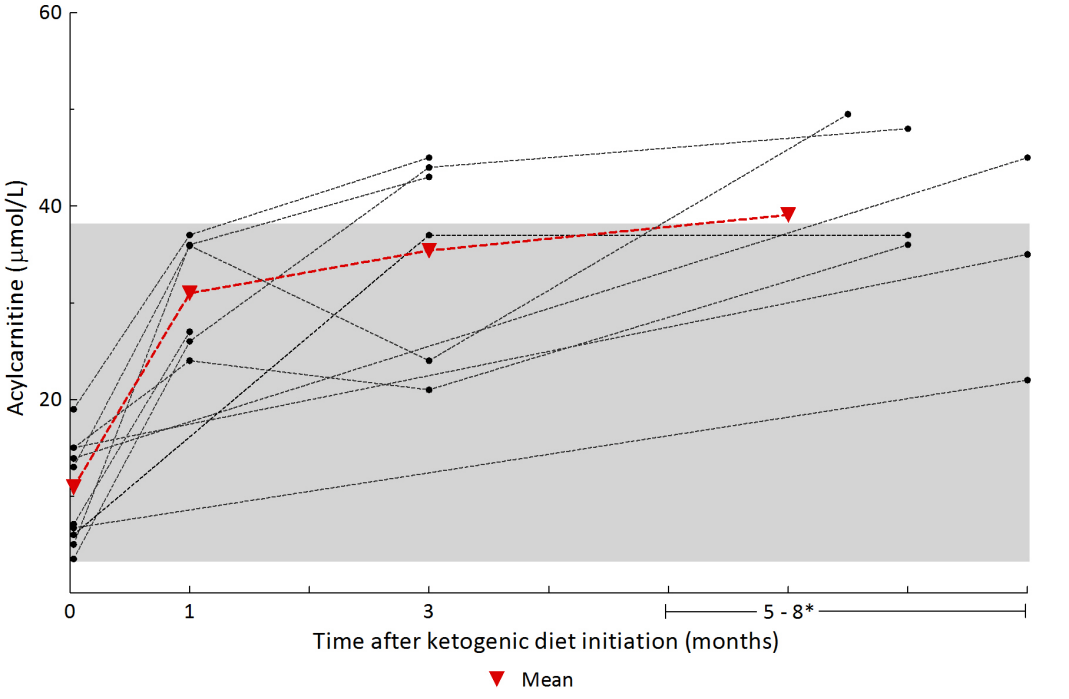
†Participants with >1 result had concentrations represented as an average (n=1)

Figure 4: Individual and mean plasma free carnitine concentrations before and after initiation of ketogenic diet treatment



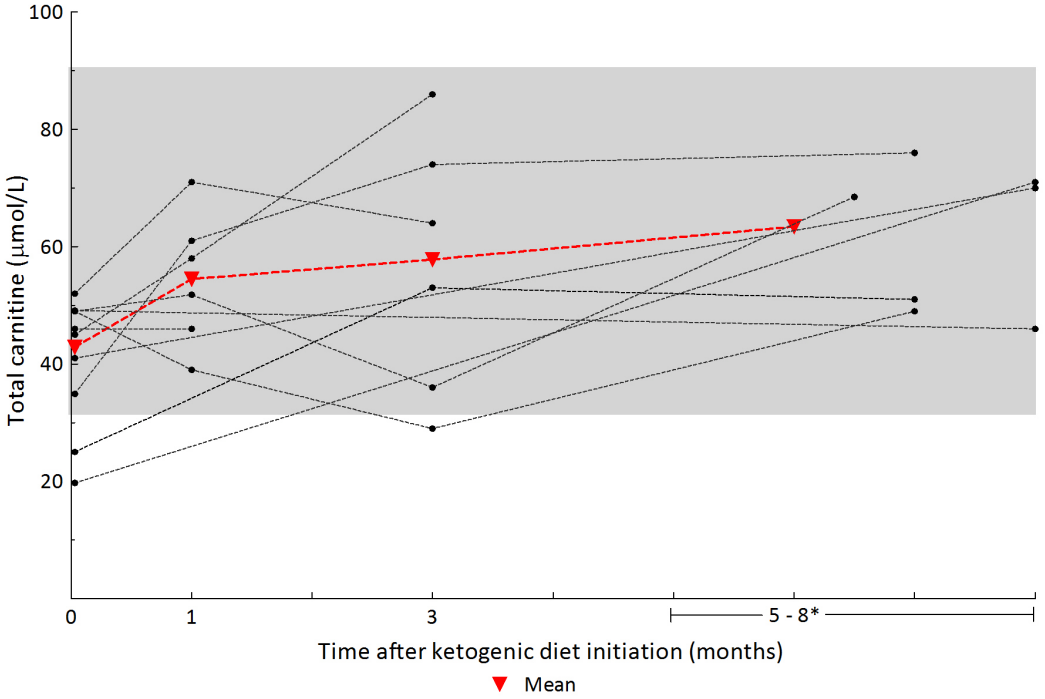
*Participants with >1 result had concentrations represented as an average (n=1)
Shaded area indicates normal reference range

Figure 5: Individual and mean plasma acylcarnitine concentrations before and after initiation of ketogenic diet treatment



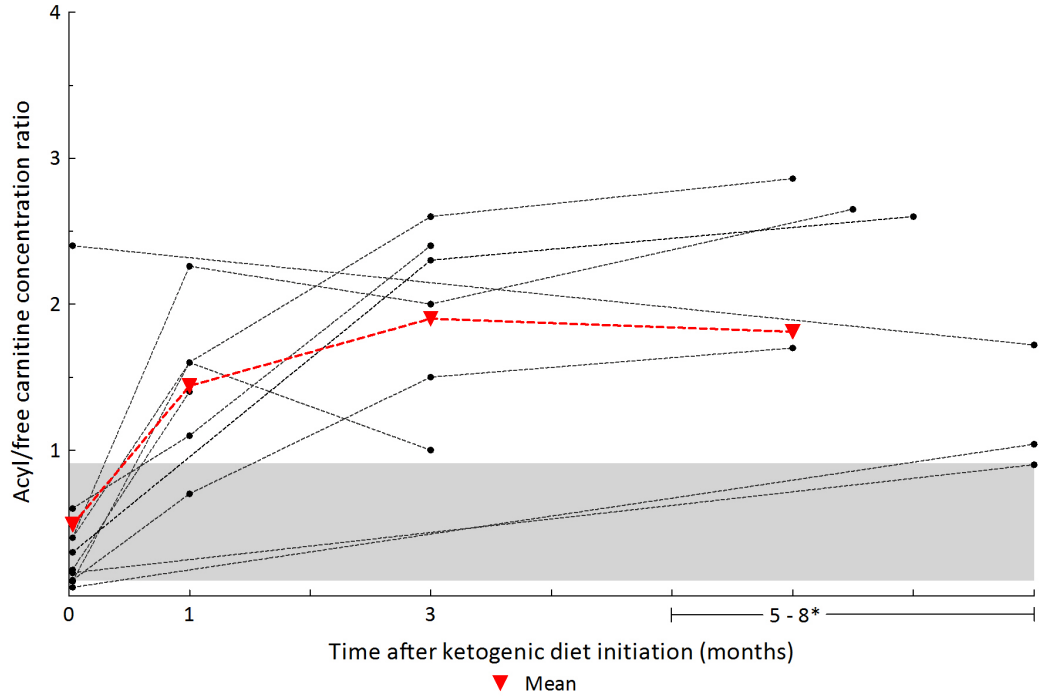
*Participants with >1 result had concentrations represented as an average (n=1)
Shaded area indicates normal reference range

Figure 6: Individual and mean plasma total carnitine concentrations before and after initiation of ketogenic diet treatment



*Participants with >1 result had concentrations represented as an average (n=1)
Shaded area indicates normal reference range

Figure 7: Individual and mean plasma acyl/free carnitine ratio before and after initiation of ketogenic diet treatment



*Participants with >1 result had concentrations represented as an average (n=1)
Shaded area indicates normal reference range

treatment. The exception was 1 participant who dropped below the normal reference range at 3 months.

Individual and mean plasma acyl/free carnitine concentration ratios before and after initiation of ketogenic diet treatment are illustrated in Figure 7. All participants but 1 had plasma acyl/free carnitine concentration ratios within the normal reference range at baseline. Those 6 participants with plasma acyl/free carnitine concentration ratios within the normal reference range at baseline all experienced plasma acyl/free carnitine concentration ratios above the normal reference range between 1 – 8 months of treatment. The single participant with a high acyl/free carnitine concentration ratio at baseline concurrently had very low free carnitine concentrations at that time point, causing the ratio of acyl/free carnitine concentrations to be above the normal reference range.

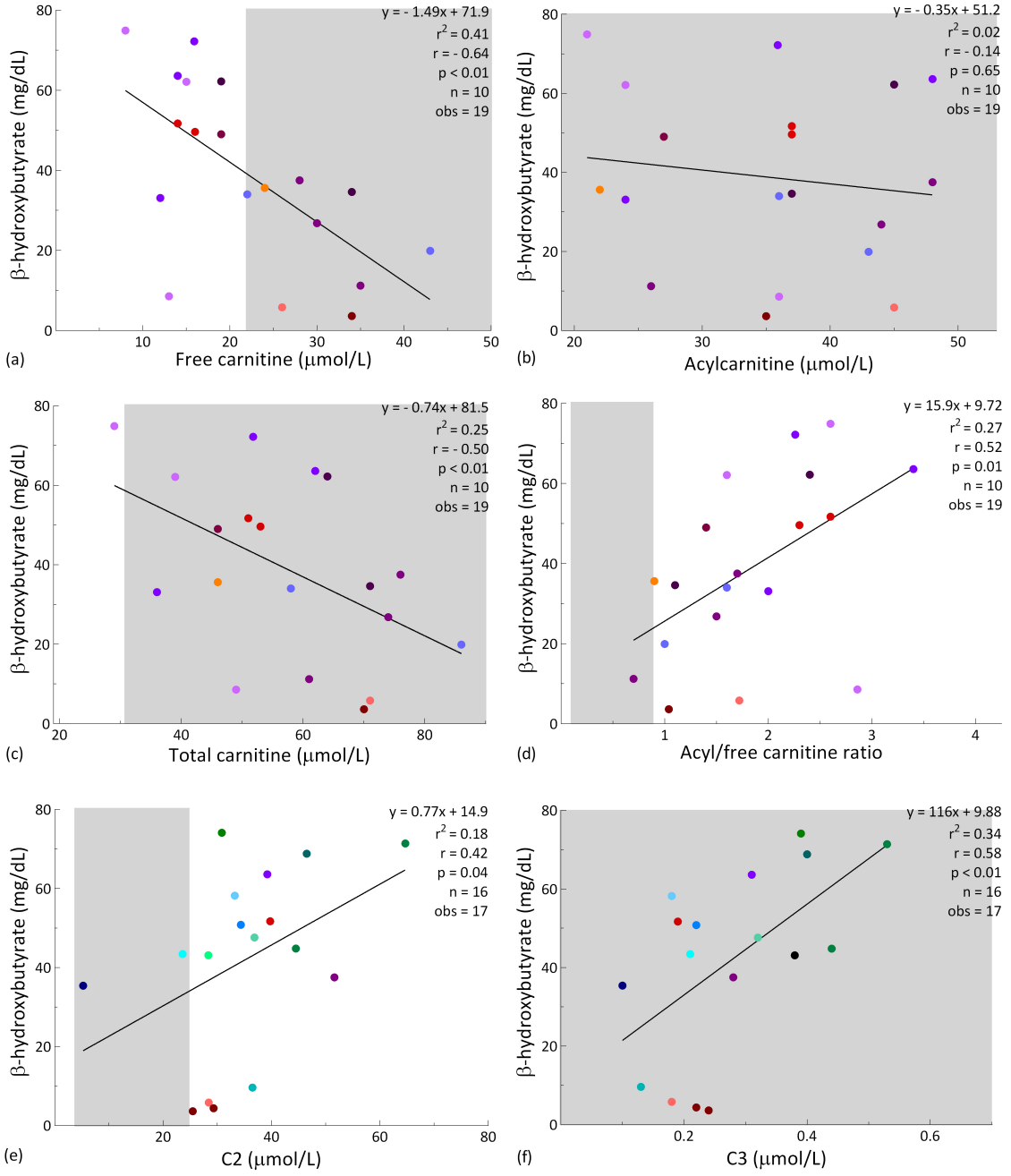
Associations between carnitine and β-hydroxybutyrate concentrations

The second aim of this research was to determine the association between plasma carnitine concentration and the degree of ketosis, measured by plasma β-hydroxybutyrate concentration, in pediatric patients with intractable epilepsy treated with the ketogenic diet. Correlations between plasma β-hydroxybutyrate concentration and plasma free carnitine, acylcarnitine, and total carnitine concentrations, acyl/free carnitine concentration ratio, and acylcarnitine species concentrations from 1 – 8 months of treatment are illustrated in Figure 8a – f.

Significant negative correlations were observed for plasma β-hydroxybutyrate concentration and plasma free ($p=0.00$) and total ($p<0.01$) carnitine concentrations. Significant positive correlations were observed for plasma β-hydroxybutyrate concentration and plasma acyl/free carnitine concentration ratio ($p=0.01$), as well as for

plasma β-hydroxybutyrate concentration and plasma concentrations of the acylcarnitine species C2 (p=0.04) and C3 (p<0.01). There were no significant correlations between plasma β-hydroxybutyrate concentration and plasma acylcarnitine concentration for

Figure 8: Correlation between plasma β-hydroxybutyrate concentration and plasma carnitine and acylcarnitine species concentrations from 1-8 months after initation of ketogenic diet treatment



Shaded area indicates normal reference range; colors indicate individual participants

any other acylcarnitine species.

Carnitine concentration and effect on treatment measures

Our final aim was to determine whether plasma carnitine concentration was associated with reduction in seizure frequency and/or number of AEDs prescribed in pediatric participants with intractable epilepsy treated with the ketogenic diet. Reduction in seizure frequency with respect to plasma free carnitine, acylcarnitine, and total carnitine concentrations and plasma acyl/free carnitine concentration ratio during 1 – 8 months of ketogenic diet treatment is described in Table 11. Those participants with a plasma acyl/free carnitine concentration ratio of at least 1.5 were approximately 10 times more likely to achieve at least a 50% reduction in seizure frequency than those with a plasma acyl/free carnitine concentration ratio of less than 1.5 ($p<0.05$). No odds ratios concerning plasma free, total, or acylcarnitine concentrations were statistically significant. Analysis performed with respect to carnitine status ("above", "within", or "below" the normal reference range) failed to produce any statistically significant odds ratios.

Reduction in the number of antiepileptic drugs prescribed with respect to plasma free carnitine, acylcarnitine, and total carnitine concentrations and plasma acyl/free carnitine concentration ratio during 3 – 8 months of ketogenic diet treatment

Table 11: Odds ratios for plasma carnitine concentration and likelihood of achieving at least a 50% reduction in seizure frequency during 1 – 8 months of ketogenic diet treatment (n=10)

Parameter	Comparison	OR	95% CI	P value	Obs
Free carnitine	>15 µmol/L vs. <15 µmol/L	0.89	0.12 – 6.62	0.91	19
Acylcarnitine	>35 µmol/L vs. <35 µmol/L	1.60	0.14 – 18.7	0.71	19
Total carnitine	>50 µmol/L vs. <50 µmol/L	1.60	0.07 – 35.6	0.77	19
Acyl/free carnitine ratio	≥1.5 vs. <1.5	10.0	1.05 – 95.2	<0.05	19

Table 12: Odds ratios for plasma carnitine concentration and likelihood of withdrawing at least one antiepileptic drug during 3 – 8 months of ketogenic diet treatment (n=9)

Parameter	Comparison	OR	95% CI	P value	Obs
Free carnitine	>15 µmol/L vs. <15 µmol/L	--*	--	--	--
Acylcarnitine	>35 µmol/L vs. <35 µmol/L	1.33	0.09 – 19.7	0.83	14
Total carnitine	>50 µmol/L vs. <50 µmol/L	0.78	0.04 – 14.3	0.87	14
Acyl/free carnitine ratio	≥1.5 vs. <1.5	9.0	0.39 – 205	0.17	14

*Analysis not statistically possible due to insufficient data

is described in Table 12. There was no statistically significant relationship between likelihood of reducing the number of AEDs prescribed by at least one medication and plasma concentrations of plasma free carnitine, acylcarnitine, and total carnitine or plasma acyl/free carnitine concentration ratio. Analysis performed with respect to carnitine status ("above", "within", or "below" the normal reference range) failed to produce any statistically significant odds ratios.

Determination of hypotheses

Our first hypothesis was that mean plasma free and total carnitine concentrations would be significantly lower, mean plasma acylcarnitine concentration and acyl/free carnitine concentration ratio would be significantly higher, and mean plasma acylcarnitine species concentrations would reflect ketosis (elevated concentrations of C2 and C4-OH acylcarnitine species) at 1, 3, and 6 months after initiating the ketogenic diet. This hypothesis was accepted in part only. We accept that mean plasma free carnitine concentration was indeed lower than baseline after initiation of the ketogenic diet (although this was not significant for the largest cohort), mean plasma acylcarnitine concentration and mean acyl/free carnitine concentration ratio were significantly higher, and that mean plasma acylcarnitine species

concentrations reflected ketosis (elevated concentrations of C2 and C4-OH acylcarnitine species) after initiation of ketogenic diet treatment. Mean plasma total carnitine concentration was actually higher than baseline after initiation of the ketogenic diet; this portion of the hypothesis was rejected.

Our second hypothesis was that plasma free carnitine, acylcarnitine, and total carnitine concentrations, and acyl/free carnitine concentration ratio would be positively correlated with plasma β-hydroxybutyrate concentration at 1, 3, and 6 months after initiating the ketogenic diet. This hypothesis was accepted in part only. Plasma free and total carnitine concentrations were in fact significantly negatively correlated with plasma β-hydroxybutyrate concentration during ketogenic diet treatment. Plasma acylcarnitine concentrations were not correlated with plasma β-hydroxybutyrate concentration. We reject these portions of the hypothesis. We accept that plasma acyl/free carnitine concentration ratio was positively correlated with plasma β-hydroxybutyrate concentration during ketogenic diet treatment.

Our final hypothesis was that patients with higher plasma free carnitine, acylcarnitine, and total carnitine concentrations and acyl/free carnitine concentration ratio would be more likely to demonstrate a reduction in seizure frequency and number of AEDs required at 1, 3, and 6 months after initiating the ketogenic diet. This hypothesis was accepted in part only. Increased plasma acyl/free carnitine concentration ratio was the only parameter to demonstrate a relationship with regard to increased likelihood of seizure reduction. We accept this portion of the hypothesis. Plasma free, total, and acylcarnitine concentration demonstrated no relationship with the likelihood of at least a 50% reduction in seizure frequency. Similarly, there was no

relationship between any carnitine parameter and the likelihood of withdrawing at least one antiepileptic drug. We reject these portions of the hypothesis.

CHAPTER 6

Discussion

Childhood epilepsy represents a significant public health concern today. Children with epilepsy face many medical challenges, of which failure to control seizures and the difficulty of managing medication-related side effects both play a serious role in determining quality of life. The ketogenic diet is unique in that it offers children with medically refractory epilepsy a better likelihood of improved seizure control than many medications without adding to the drug burden of these patients. The ketogenic diet has been proven both safe and effective; however, any measure of improvement on the efficacy of or tolerance to this diet could provide additional relief for children who continue to struggle with recurrent or break-through seizures.

Although the exact mechanism by which the ketogenic diet reduces seizures is unknown, increased fatty acid oxidation and subsequent ketosis have been established as essential for effective ketogenic diet therapy. As described previously in this text, the oxidation of LCTs (the majority of ingested dietary fat) and resultant production of ketones first requires adequate free carnitine to facilitate fatty acid import into the mitochondria via the carnitine shuttle. For this reason, some clinicians have suggested that carnitine supplementation might enhance fatty acid oxidation and ketosis, and therefore may potentially improve seizure control.

Before the scientific community can assess whether supplemental carnitine is beneficial to children treated with the ketogenic diet for epilepsy, first we must verify the existence and course of changes in carnitine status observed during ketogenic diet treatment. The impact of the ketogenic diet on carnitine in the absence of supplementation is a crucial puzzle piece that has remained inconclusive.

Significance of changes to carnitine concentration

We observed that the ketogenic diet significantly impacted plasma free carnitine, acylcarnitine, and total carnitine concentrations and acyl/free carnitine concentration ratio during the first 8 months of ketogenic diet treatment. This impact was demonstrated by: differences in mean plasma concentration between time points, overall shared trends in individual plasma concentrations during treatment, and changes to individual status (determined as "above," "within," or "below" normal reference ranges for the parameters) during treatment.

We discerned that it was relatively common for participants in this study to experience plasma free carnitine concentrations below the normal reference range during the first 8 months of ketogenic diet treatment. Mean plasma free carnitine concentration was marginally within the normal reference range from 1 – 8 months of ketogenic diet treatment. Lowest mean plasma free carnitine concentration occurred at 3 months after ketogenic diet initiation, with a slight upward trend in mean plasma concentration observed at 5 – 8 months. It appears that plasma free carnitine concentration was reduced by the ketogenic diet at least in part due to the increased formation of acylcarnitine, which reflected a dramatically increased rate of fatty acid oxidation and therefore increased need for fatty acid transport into the mitochondria.

Our results regarding plasma free carnitine concentration during ketogenic diet therapy contradict the findings of Coppola, et al., who assessed plasma free carnitine concentrations in 11 pediatric patients treated by ketogenic diet in 2006. Coppola, et al., at concluded that there was "no evidence of abnormal free carnitine concentrations" for their participants at baseline or 3 months after ketogenic diet initiation (53). This may be due to the fact that Coppola, et al., defined abnormal plasma free carnitine concentration as less than 20 μmol/L, the concentration that has been historically cited as a potential benchmark for secondary carnitine deficiency. In contrast, we defined the normal reference range for plasma free carnitine concentration as 22 – 63 μmol/L according to current age-specific reference ranges. Of note, mean plasma carnitine concentration before and during ketogenic diet treatment for the participants included by Coppola, et al., was 21.17 ± 8.69 μmol/L at baseline and 22.03 ± 9.65 μmol/L at 3 months after ketogenic diet initiation. These mean concentrations are first below and then very marginally within the reference range established for our biochemical analysis. Employing a lower bound of 20 μmol/L for our biochemical analysis of plasma free carnitine concentration would not have appreciably changed our results.

Mean plasma acylcarnitine concentration demonstrated a marked increase from baseline throughout the duration of ketogenic diet treatment for participants included in our study. The same overall trend was exhibited by all individual participants, with more than 50% of participants achieving plasma concentrations above the normal reference range between 3 – 8 months of ketogenic diet treatment. As stated above, the rise in plasma acylcarnitine concentration suggests a dramatic increase in the rate of

fatty acid oxidation and the formation of acylcarnitine for subsequent transport of fatty acids out of the mitochondria.

Mean plasma total carnitine concentration exhibited a general upward trend during the first 8 months of ketogenic diet treatment for the participants of this study, with mean plasma total carnitine concentration remaining within the normal reference range for the duration of treatment. We suggest that plasma total carnitine concentration is increased during ketogenic diet treatment primarily as a reflection of the observed large increase in acylcarnitine concentration. It is also possible that increased fatty acid oxidation stimulates endogenous carnitine synthesis in children on the ketogenic diet, thus increasing plasma total carnitine concentrations. Although this physiological process has not yet been established in humans, it has been observed in rats. Rigault, et al., reported an increase in transcription of BBOX1 (the gene that controls expression of γ-butyrobetaine hydroxylase, the final enzyme in the carnitine biosynthesis pathway) leading to notably increased carnitine concentrations in the blood, liver, and muscle of rats fed a high-fat diet for 4 weeks as compared to control animals (60).

Our findings contradict results observed by Berry-Kravis, et al., who described mean plasma total carnitine concentrations for patients treated with the ketogenic diet in 2001 (36). In contrast to the upward trend observed in our participants, Berry-Kravis, et al., observed plasma mean total carnitine concentrations significantly lower than baseline at 1 and 6 months after ketogenic diet initiation. Berry-Kravis, et al., also described plasma total carnitine concentrations indicative of deficiency occurring for 6

of 43 patients during 1 – 12 months of ketogenic diet treatment. Again, the definition of deficiency for this study differed slightly from the reference range that we chose for interpretation of our biochemical analysis. Berry-Kravis, et al., utilized a gender-specific reference range for total plasma carnitine concentrations, with deficiency defined as less than 31 μmol/L for males and less than 25 μmol/L for females.

It is of note that Berry-Kravis, et al., reviewed medical records for patients who initiated ketogenic diet treatment between 1997 – 2000. At this time, patients on the ketogenic diet primarily either consumed Ross Carbohydrate Free soy formula (Abbott Nutrition, Columbus, OH) along with modular products to meet nutritional needs or a food-based ketogenic diet (61). Ross Carbohydrate Free soy formula contains 3 mg of carnitine per 100 mL of formula (62). KetoCal (Nutricia, Gaithersburg, MD) products were introduced to the market in 2001 and include several varieties of nutritionally complete ketogenic formulas. KetoCal formulas provide 6.4 – 8.3 mg carnitine per 100 mL of formula (63). For reference, a standard complete pediatric nutritional formula such as Nutren Junior Fiber (Nestle, Vevey, Switzerland) provides 4 mg of carnitine per 100 mL of formula (64). A 4 year old child weighing 16 kg and requiring 1,050 kcal per day on the ketogenic diet might consume approximately 700 mL/day of KetoCal 4:1 Liquid, providing 58 mg of carnitine per day (3.6 mg/kg/day). This is above the expected carnitine intake for a child consuming a typical, food-based, non-therapeutic, non-protein restricted diet (0.3 – 1.9 mg/kg/day) (65). Approximately 73% of the participants included in our study were provided KetoCal formula, either as their sole source of nutrition or as an oral supplement. We propose the possibility that our participants

ingested comparatively more carnitine than those included by Berry-Kravis, et al., which may have modulated the effect of the ketogenic diet on plasma total carnitine concentrations for our participants.

Mean plasma acyl/free carnitine concentration ratio demonstrated the most dramatic increase over time. This was likely due to the simultaneous downward trend in free plasma carnitine concentrations and the pronounced upward trend in plasma acylcarnitine concentrations. All participants had plasma acyl/free carnitine concentration ratios equal to or above the upper bound of the normal reference range from 3 – 8 months of ketogenic diet treatment, with the highest individual acyl/free carnitine concentration ratio as 2.86. These results are consistent with those reported by Berry-Kravis, et al., who observed plasma acyl/free carnitine concentrations of 0.44 – 3.25 during ketogenic diet treatment (36). This confirms that an elevated acyl/free carnitine concentration ratio is likely indicative of increased fatty acid oxidation in the context of a ketogenic diet rather than carnitine deficiency as it has been historically described (37).

Significance of changes to acylcarnitine species concentrations

Mean plasma concentrations for acylcarnitine species observed in study participants after initiation of ketogenic diet treatment exhibited the characteristic peaks of acetylcarnitine (C2) and hydroxybutyrylcarnitine (C4-OH) concentrations when analyzed by tandem mass spectrometry that have been previously described by Smith, et al., as hallmarks of ketosis (66). These changes are physiologically expected. Acetylcarnitine is synthesized by esterification of acetyl-CoA to carnitine. Plasma

acetylcarnitine concentration is expected to rise during the ketogenic diet due to increased mitochondrial acetyl-CoA production; acetyl-CoA that remains in circulation (i.e., that does not enter the TCA cycle or contribute to ketone synthesis) is free to esterify to carnitine and produce increased concentrations of acetylcarnitine. Hydroxybutyrylcarnitine is synthesized by esterification of β-hydroxybutyrate, which circulates in very high concentrations during ketosis, to carnitine. For this reason, plasma hydroxybutyrylcarnitine is also expected to increase dramatically during ketogenic diet treatment.

In addition to observing increased plasma concentration of acylcarnitine species that are known indicators of ketosis, we also observed additional changes to certain other plasma acylcarnitine species concentrations. We propose that additional acylcarnitine species for which mean concentrations were significantly increased from baseline represent the specific fatty acids that were ingested and oxidized in proportionally greater amounts during ketogenic diet treatment compared to the amount that was ingested and oxidized when participants were following a typical, non-therapeutic diet. This mirrors conclusions drawn by Kien, et al., who examined the effect of high and low dietary intakes of palmitic acid and oleic acid on serum concentrations of the product acylcarnitine species for those fatty acids, and Costa, et al., who described a marked increase in polyunsaturated acylcarnitine species and a blunted increase in saturated acylcarnitine species reflective of the fatty acid composition of an orally administered loading dose of sunflower oil (51,67).

There are some limitations to our interpretation of plasma acylcarnitine species

as reflective of oxidized fatty acids. First, it must be noted that acylcarnitine species may also be synthesized through esterification of carnitine by a fatty acid that has been mobilized from adipose tissue, which would primarily occur during times of fasting or insufficient energy intake. It is impossible to clearly differentiate acylcarnitine species that are synthesized from ingested fatty acids or endogenous fatty acids in the assay used for analysis of our biochemical data. Second, medium-chain acylcarnitine species can arise from a third source: incomplete β-oxidation of long chain fatty acids. It is possible that high demand for fatty acid oxidation in the mitochondria may cause fatty acid overload, resulting in the incomplete β-oxidation of longer chain fatty acids and formation of medium-chain fatty acids as byproducts. These chain-shortened fatty acids may then exit the mitochondria via the carnitine shuttle and enter the blood as medium-chain acylcarnitines (68). A final consideration in the analysis of plasma acylcarnitine species is that fatty acids are not oxidized directly in proportion to their availability. Carnitine palmitoyltransferase I, the first enzyme in the carnitine shuttle, exhibits greatest affinity for the acyl-CoA derivatives of palmitoleic acid (16:1, ω-7) (69). This means that palmitoleylcarnitine is preferentially synthesized by CPT-1 and is expected to be completely degraded to form acetyl-CoA units; however, in fatty acid overload of the mitochondria this acylcarnitine species may be only partially oxidized and could also enter the plasma as a chain-shortened acylcarnitine species, such as tetradecenoylcarnitine (C14:1). High saturated fat diets can alter this preferential conversion of fatty acids to acylcarnitines by CPT-1; for example, CPT-1 has increased affinity for palmitic acid (16:0) (converted to palmitoylcarnitine) when a greater

proportion of dietary fats are saturated in nature (69).

Given that the children included in this study ingested 87 — 90% of their calories as dietary fat, it is likely that the profile of ingested fatty acids played a major (though not exclusive) role in determining the plasma acylcarnitine species concentrations. Participants in this study demonstrated an increase in certain medium-chain saturated and unsaturated acylcarnitine species, specifically: octanoylcarnitine (C8), decanoylcarnitine (C10), and decenoylcarnitine (C10:1). Octanoylcarnitine is synthesized by the esterification of caprylic acid (8:0) to carnitine. Caprylic acid is found in coconut-based products (coconut milk, coconut meat, and coconut oil) (70). Decanoylcarnitine is synthesized by the esterification of capric acid (10:0) to carnitine. Capric acid is found in foods such as coconut milk, feta cheese, and coconut meat (70). Smaller amounts of capric acid are also found in cheddar cheese and heavy whipping cream. Decenoylcarnitine is synthesized by the esterification of caproleic acid (10:1, ω-1) to carnitine. Caproleic acid is found primarily in butterfat.

Participants also demonstrated an increase in certain long-chain saturated and unsaturated acylcarnitine species, specifically: tetradecenoylcarnitine (C14:1), stearoylcarnitine (C18), oleylcarnitine (C18:1), and linoleylcarnitine (C18:2). Tetradecenoylcarnitine is synthesized by the esterification of myristoleic acid (14:1, ω-5) to carnitine. Myristoleic acid is found in foods such as cheddar cheese and beef (70). Stearoylcarnitine is synthesized by esterification of stearic acid (18:0) to carnitine. Stearic acid is found in foods such as cocoa powder, beef, lamb, pork, and soybean oil. Oleylcarnitine is synthesized by the esterification of oleic acid (18:1, ω-9) to carnitine.

Oleic acid is found in a variety of foods, including: macadamia nuts, almonds, hazelnuts, pecans, peanuts, palm oil, canola oil, olive oil, avocados, soybean oils, and pork. Linoleylcarnitine is synthesized by esterification of linoleic acid (18:2, ω-6) to carnitine. Linoleic acid is found in foods such as sunflower seeds, walnuts, brazil nuts, safflower oil, canola oil, and soybean oil (70).

Many children included in this study were fed with a specialty nutritional formula, either in combination with recreational oral intake or as the sole source of nutrition provided. For children who received the majority of their nutrition from nutritional formula, we infer that plasma acylcarnitine concentrations were likely influenced primarily by the types of fat included in the formula provided. KetoCal formulas were most commonly prescribed for the participants of this study. Of the energy provided by KetoCal products, 87.1 – 88.7% is derived from fat. The primary source of fat in KetoCal products is refined vegetable oil, specifically palm, soy, and sunflower (both high oleic and unspecified) oils (63). A child consuming these oils would ingest a high proportion of stearic acid, oleic acid, and linoleic acid, producing higher plasma concentrations of stearoylcarnitine, oleylcarnitine, and linoleylcarnitine. This is consistent with the acylcarnitine species identified as having significantly increased concentrations from baseline after ketogenic diet initiation for our participants.

We suggest that the participants who did initiate and maintain food-based ketogenic diet treatment or who consumed a considerable amount of their daily energy from food in addition to nutritional formula may have increased their consumption of coconut products, cheese, heavy whipping cream, and butter as reflected by the

changes in plasma medium-chain acylcarnitine species concentrations observed in our sample. We also suggest that these participants may have increased their consumption of cheese, nuts, seeds, oils, and avocado, as reflected by the changes in plasma long-chain acylcarnitine species concentrations observed in our sample. It is unlikely that meat consumption contributed greatly to the acylcarnitine concentrations of participants due to the protein-restricted nature of the ketogenic diet.

It is of note that palmitoylcarnitine (C16) was not significantly increased from baseline at 5 – 8 months after ketogenic diet initiation. Palmitoylcarnitine is synthesized by esterification of palmitic acid to carnitine. Palmitic acid is one of the most common dietary saturated fatty acids and is found in cheese, meats, dairy products, and nuts. It is likely that palmitic acid was already consumed in moderate amounts by children following a regular diet prior to ketogenic diet initiation, and it is also likely that palmitic acid continued to be consumed after ketogenic diet initiation. It is possible that the lack of a notable increase in consumption of palmitic acid was responsible for the unremarkable effect on plasma palmitoylcarnitine concentrations.

Examination of the relationship between ketosis and carnitine

Plasma β-hydroxybutyrate concentration exhibited a significant negative correlation with plasma free and total carnitine concentrations. Although the negative correlations between circulating plasma β-hydroxybutyrate concentration and plasma free and total carnitine concentrations was unexpected, we propose the possible explanation that a higher rate of fatty acids oxidation could generate 1) proportionally more plasma acylcarnitine produced by increased fatty acid transport into the

mitochondria and 2) greater plasma concentration of β-hydroxybutyrate. This would result in consequentially lower plasma concentrations of free carnitine in concurrence with appropriate plasma concentrations of β-hydroxybutyrate. It is known that plasma carnitine is filtered into the glomerular filtrate and excreted by the kidneys when it occurs in high concentrations, and that renal reabsorption of carnitine begins to decline at plasma concentrations above 40 – 60 μmol/L (33,71). This could also lead to consequentially lower plasma total carnitine concentrations, again in concurrence with appropriate plasma concentrations of β-hydroxybutyrate. This hypothesis is weakened by the fact that plasma acylcarnitine concentration failed to exhibit a significant correlation with plasma β-hydroxybutyrate concentration; however, it is strengthened by the fact that plasma β-hydroxybutyrate concentration did display a strong positive correlation with plasma acyl/free carnitine concentration ratio. This relationship between plasma β-hydroxybutyrate concentration and plasma acyl/free carnitine concentration ratio also strengthens our earlier assertion that plasma acyl/free carnitine concentration ratio should be considered as a potential measure of fatty acid oxidation and perhaps indirectly of the strength of ketosis.

Significant positive correlations were observed between plasma β-hydroxybutyrate concentration and plasma acetylcarnitine (C2) and propionylcarnitine (C3) concentrations. The positive correlation between plasma β-hydroxybutyrate and plasma acetylcarnitine concentration was expected because 1) acetylcarnitine is the product of acetyl-CoA esterified to carnitine, and 2) acetyl-CoA is the primary substrate for the formation of the parent ketone body acetoacetate, which is rapidly converted to

β-hydroxybutyrate by the enzyme β-hydroxybutyrate dehydrogenase.

The positive correlation between plasma β-hydroxybutyrate and plasma propionylcarnitine concentration is explained by the facts that 1) elevated concentrations of propionylcarnitine have been established to reflect increased branched-chain amino acid (BCAA) accumulation, and 2) the BCAA leucine has been proposed by recent literature to be ketotic in nature (72,73). In fact, Evangeliou, et al., recently published data regarding a trial of BCAA supplementation during ketogenic diet therapy that resulted in no detrimental impact on plasma β-hydroxybutyrate concentrations despite a lowered ketogenic diet ratio (from 4:1 to 2.5:1) as a result of the additional grams of protein consumed (74). The source of BCAA accumulation suggested by increased plasma propionylcarnitine concentrations in patients treated by the ketogenic diet who are not supplemented with BCAAs is unclear; potential sources include food (primary sources of BCAAs are identified as meat, eggs, soybeans and soy-based products, cheese, and powdered milk), nutritional formula (KetoCal formulas contain both powdered milk protein and soy fiber), or skeletal muscle catabolism (63,70,73).

It must be noted that the conclusions of this study are derived solely from participants not prescribed supplemental carnitine, and who demonstrated an equal tendency to either remain within or drop below the normal reference range for free carnitine. These results may or may not be applicable to a participant population with free carnitine concentrations within the normal reference range.

Interpretation of the effect of carnitine on treatment measures

The sole carnitine parameter that demonstrated a statistically significant relationship with seizure reduction during ketogenic diet treatment was plasma acyl/free carnitine concentration ratio. Participants with a plasma acyl/free carnitine concentration ratio of at least 1.5 were approximately 10 times more likely to achieve at least a 50% reduction in seizure frequency compared to those with a ratio of less than 1.5. We have suggested earlier in this text that acyl/free carnitine concentration ratio may serve as an indicator of rate of fatty acid oxidation and therefore as a surrogate marker for strength of ketosis. However, it is of note that exploratory analysis of plasma β-hydroxybutyrate concentrations and likelihood of achieving at least a 50% reduction in seizure frequency failed to produce any statistically significant odds ratios whatsoever. This implies that seizure reduction may be a benefit of the change in primary energy-producing substrate from glucose to fatty acids, and that this change may be the primary driver of seizure reduction rather than circulating ketone concentrations. This also suggests that plasma acyl/free carnitine concentration ratio may be of more use than simply as a surrogate marker of ketogenesis. Further research is needed to solidify the relationship between a higher plasma acyl/free carnitine concentration ratio and potentially increased likelihood for seizure reduction.

Plasma free carnitine, acylcarnitine, and total carnitine concentrations demonstrated no statistically significant relationships with the likelihood of at least a 50% reduction in seizure frequency. Similarly, there was no relationship between any carnitine parameter and likelihood of withdrawing at least one antiepileptic drug. This is

likely because withdrawal of antiepileptic drugs is driven by very different factors than seizure reduction. For the participants of this study, changes to antiepileptic drug regimens were made according to the comfort and wishes of the family in accordance with medical recommendations of the supervising physician. It is not uncommon for parents of children with intractable epilepsy who are treated with ketogenic diet therapy to be reluctant to withdraw medications, even if seizure control begins to improve; this is because they are concerned that any new changes they make might negatively impact their child's newfound improvement in seizure frequency.

Study strengths

Strengths of this study included the many different measures of carnitine that were assessed for these participants over time. Previously studies have only assessed one or two parameters for plasma carnitine (36,53). In contrast, we assessed four main categories of carnitine measurement (plasma free carnitine, acylcarnitine, and total carnitine concentrations and plasma acyl/free carnitine concentration ratio) and further assessed derivatives of acylcarnitine by examining the many acylcarnitine species that are synthesized by the esterification of fatty acids to carnitine. This allowed us to generate a more complete picture of the changes to carnitine that occur during ketogenic diet treatment than previous research has accomplished.

Statistical analysis for this study was strengthened by our analysis of multiple cohorts of participants according to their available data. We examined both longitudinal and cross-sectional data to interpret changes in plasma free carnitine, acylcarnitine, and total carnitine concentrations and acyl/free carnitine concentration ratio. This provided

additional statistical support for our evidence and conclusions.

Thirdly, we employed a very comprehensive medical record review to accomplish this research. This allowed us to describe our patient population in detail and to thoroughly explore any possible confounding factors for the conclusions that we stated. In addition, our meticulous investigation of our study population allows us to confidently say that our participants were reflective of those included by many other published research studies of pediatric patients with epilepsy treated by the ketogenic diet. Because of this, our results should be appropriately generalizable to other pediatric patients with intractable epilepsy.

Study limitations

The primary limitation of this research was our relatively small sample size. Our original intent was to include only prospectively followed patients in our medical record review, which would have allowed us to collect data for children who were managed by the same two providers and who followed the same biochemical analysis protocol for the duration of their treatment. We anticipated 12 – 24 children to fall within this eligibility based on prior ketogenic diet initiation rates at the Doernbecher Children's Hospital Ketogenic Diet Clinic. One unforeseen factor that limited our sample size included a recent trend towards the modified Atkins diet treatment as an attempted first pass therapy prior to initiating full ketogenic diet. Modified Atkins patients do not receive laboratory analysis at the Ketogenic Diet Clinic and therefore this prevented assessment of a true biochemical baseline for these patients and required their exclusion from the study.

Providers at the ketogenic diet clinic also encountered a situational reluctance of parents to initiate ketogenic diet during the autumn and winter holidays, which resulted in delayed ketogenic diet initiations for multiple participants and therefore limited the number of follow-ups they were able to attend during the course of our medical record review. As described earlier, participants who did not have at least two visits with biochemical data available at the time of medical record review were not able to be included.

Patient adherence to biochemical assessment protocols was an additional limitation. Missed or cancelled follow-up visits contributed further to inconsistencies within the data set and also limited the number of participants included in this study. Medical records of patients who did not meet our minimum requirement for biochemical data due to missed or cancelled follow-up appointments were not eligible for inclusion in this study.

Because of these challenges, we chose to include retrospectively reviewed medical records in our research. This resulted in the inclusion of children who were medically managed by several different pediatric epilepsy specialists, each of whom provided different Standards of Care according to their clinical judgment and personal preference. This caused additional inconsistencies with regard to the laboratory protocols that different participants followed during ketogenic diet treatment.

Another limitation for the biochemical data assessed in this study was that we discovered considerable variation in the acylcarnitine species reported for participants by different laboratories. Similar variation was also evident according to the time frame

in which the assessment was performed. Acylcarnitine species analysis performed prior to 2013 had both additional and missing acylcarnitine species reported when compared to the acylcarnitine species commonly reported today. Another concern relative to the several different laboratories that performed acylcarnitine species analysis is that these laboratories employed different reference ranges, which implies different degrees of sensitivity for their biochemical processing equipment even if assays were performed according to the same method.

A limitation we experienced that is common to epilepsy-related research is that we relied upon parent report (as noted in the medical record) to measure seizure frequency for participants before and during ketogenic diet treatment. Because seizures are an experience for parents and not a data point as they are for researchers, it can be difficult for providers to extract a clear description of seizure frequency that can then be reported in the medical record. Also, parents are not always with children; parents must rely on second-hand reports of seizures from teachers and caregivers when children are at school or in daycare. Providers for the participants of this study documented seizure frequency from parent report as clearly and faithfully as possible.

A final limitation of this research is that we studied plasma carnitine concentrations solely in children who were not prescribed supplemental carnitine. Our results may not be generalizable to children who are prescribed supplemental carnitine. Further research is needed to determine whether similar findings occur in children who receive pharmacological doses of supplemental carnitine.

Clinical implications

This study clarifies that there are demonstrated impacts of the ketogenic diet on

plasma carnitine concentration for children with intractable epilepsy who are not prescribed supplemental carnitine, particularly with respect to reduced plasma free carnitine concentration. However, we also observed an upward trend in plasma total carnitine concentration during treatment as a result of increased plasma acylcarnitine concentration, which suggests that depletion of plasma total carnitine concentration may not be common during ketogenic diet treatment. We suggest that plasma acylcarnitine concentration and acyl/free carnitine concentration ratio are commonly and appropriately above the normal reference range during ketogenic diet treatment. Additionally, plasma acyl/free carnitine concentration ratio may be a helpful parameter for providers to assess rate of fatty acid oxidation, strength of ketosis, and potentially compliance to ketogenic diet therapy. Plasma acyl/free carnitine concentration ratio may also potentially be a marker for likelihood of achieving seizure reduction during ketogenic diet therapy. Assessment of plasma free carnitine, acylcarnitine, and total carnitine concentrations and acyl/free carnitine concentration ratio should be performed on a regular basis during ketogenic diet therapy to assess for carnitine deficiency and to collect additional clinical information if desired.

This research suggests that low plasma free carnitine concentration does not appear to significantly limit ketogenesis when occurring in tandem with appropriate concentrations of total carnitine and acylcarnitine. Specific acylcarnitine species are known to demonstrate changes in plasma concentration during ketosis and may provide additional information regarding the kinds of dietary fatty acids consumed if this information is of clinical interest to providers.

CHAPTER 7

Summary and Conclusions

We conducted a medical record review to examine the impact of the ketogenic diet on plasma carnitine concentration in children with intractable epilepsy and to assess the potential associations between plasma carnitine concentration and response to ketogenic diet therapy. We conclude that the ketogenic diet significantly impacts plasma carnitine concentrations during the first 8 months of ketogenic diet treatment, specifically as: reduced plasma free carnitine concentration, increased plasma total and acylcarnitine concentrations, increased plasma acyl/free carnitine concentration ratio, and increased plasma acylcarnitine species concentrations of: C2, C4-OH, C8, C10, C10:1, C14:1, C18, C18:1, and C18:2.

We suggest that increased degree of ketosis is associated with lower plasma free and total carnitine concentrations and with higher plasma acyl/free carnitine concentration ratios, as well with higher plasma acylcarnitine species concentrations of C2 (acetylcarnitine) and C3 (propionylcarnitine).

We determine that children with plasma acyl/free carnitine concentration ratios of at least 1.5 may be more likely to experience at least a 50% reduction in seizure frequency. We do not find any evidence suggesting that likelihood of AED withdrawal is dependent on plasma carnitine concentrations or status.

Future directions

One possibility moving forward is to continue to follow the participants of this study in order to collect and analyze additional data. Another potential option would be to explore the possibility of a clinical trial of L-carnitine supplementation for pediatric patients with intractable epilepsy who are treated by ketogenic diet therapy. This current study could effectively serve as a historical control for such research. An exploration of carnitine supplementation would allow us to assess whether low plasma free carnitine concentration was a limiting factor for any of the results derived from this study.

REFERENCES

1. Hauser WA, Annegers JF, Kurland LT. Incidence of epilepsy and unprovoked seizures in Rochester, Minnesota: 1935–1984. Epilepsia 1993;34:453-68.

2. National Survey of Children's Health. Data query from the Child and Adolescent Health Measurement Initiative, Data Resource Center for Child and Adolescent Health website. Retrieved [8/20/2014] from www.childhealthdata.org.

3. Berg AT, Shinnar S, Levy SR, et al. Early development of intractable epilepsy in children: A prospective study. Neurology 2001;56:1445-52.

4. Berg AT, Vickrey BG, Testa FM, Levy SR, Shinnar S, DiMario F, Smith S. How long does it take for epilepsy to become intractable? A prospective investigation. Ann Neurol 2006;60:73-9.

5. Neal EG, Chaffe H, Schwartz RH, et al. The ketogenic diet for the treatment of childhood epilepsy: a randomised controlled trial. Lancet Neurol 2008;7:500-6.

6. Vining EP, Freeman JM, Ballaban-Gil K, et al. A multicenter study of the efficacy of the ketogenic diet. Arch Neurol 1998;55:1433-7.

7. Freeman JM, Vining EP, Kossoff EH, et al. A blinded, crossover study of the efficacy of the ketogenic diet. Epilepsia 2009;50:322-5.

8. Wilder RM. The effect on ketonemia on the course of epilepsy. Mayo Clin Bull 1921;2:307.

9. Peterman M. The ketogenic diet in epilepsy. JAMA 1925;84(26):1979-83.

10. Hemingway C, Freeman JM, Pillas DJ. The ketogenic diet: a 3- to 6-year follow-up of 150 children enrolled prospectively. Pediatrics 2001;108:898-905.

11. Paulson DJ, Hoganson GE, Traxler J, et al. Ketogenic effects of carnitine in patients with muscular dystrophy and cytochrome oxidase deficiency. Biochem Med and Metab Biol 1988;39:40-7.

12. Kossof EH, Zupec-Kania BA, Amark PE, et al. Optimal clinical management of children receiving the ketogenic diet: recommendations of the international ketogenic diet study group. Epilepsia 2009;50:304-17.

13. Thurman DJ, Beghi E, Begley CE, et al, and the ILAE Commission on Epidemiology. Standards for epidemiologic studies and surveillance of epilepsy. Epilepsia 2011;52(suppl 7):2-26.

14. Bergqvist AGC, Schall JI, Gallagher PR, Cnaan A, Stallings VA. Fasting versus Gradual Initiation of the Ketogenic Diet: A Prospective, Randomized Clinical Trial of Efficacy. Epilepsia 2005;46:1810-9.

15. Kim DW, Kang HC, Park JC, et al. Benefits of the Nonfasting Ketogenic Diet Compared With the Initial Fasting Ketogenic Diet. Pediatrics 2004;114(6):1627-30.

16. Kossoff EH, McGrogan JR, Bluml RM, Pillas DJ, Rubenstein JE, Vining EP. A Modified Atkins Diet Is Effective for the Treatment of Intractable Pediatric Epilepsy. Epilepsia 2006;47:421-4.

17. Muzykewicz DA, Lyczkowski DA, Memon N, Conant KD, Pfeifer HH, Thiele EA. Efficacy, safety, and tolerability of the low glycemic index treatment in pediatric epilepsy. Epilepsia 2009;50:1118-26.

18. Kossoff EH, Pyzik PL, McGrogan JR, Rubenstein JE. The impact of early versus late anticonvulsant reduction after ketogenic diet initiation. Epilepsy & Behavior 2004;5:499-502.

19. Kothare SV, Kaleyias J. The adverse effects of antiepileptic drugs in children. Expert Opin Drug Saf 2007;6:251-65.

20. Nettekoven S, Ströhle A, Trunz B. Effects of antiepileptic drug therapy on vitamin D status and biochemical markers of bone turnover in children with epilepsy. Eur J Pediatr 2008;167:1369-77.

21. Linnebank M, Moskau S, Semmler A, Widman G, Stoffel-Wagner B, Weller M, Elger CE. Antiepileptic drugs interact with folate and vitamin B12 serum levels. Ann Neurol 2011;69:352-9.

22. Tamura T, Aiso K, Johnston KE, et al. Homocysteine, folate, vitamin B-12 and vitamin B-6 in patients receiving antiepileptic drug monotherapy. Epilepsy Res 2000;40(1):7-15.

23. Sener U, Zorlu Y, Karaguzel O, et al. Effects of common anti-epileptic drug monotherapy on serum levels of homocysteine, Vitamin B12, folic acid and Vitamin B6. Seizure 2006;15(2):79-85.

24. Chapter 3: Neuropharmacology of Antiepileptic Drugs. In: Bromfield EB, Cavazos JE, Sirven J, ed. An Introduction to Epilepsy. West Hartford (CT): American Epilepsy Society, 2006:

25. Perucca P GF. Adverse effects of antiepileptic drugs. Lancet Neurol 2012;11(9):792-802.

26. Kang HC, Chung DE, Kim DW, Kim HD. Early- and Late-onset Complications of the Ketogenic Diet for Intractable Epilepsy. Epilepsia 2004;45:1116-23.

27. Chapter 20: Carnitine. In: Neal E, ed. Dietary Treatment of Epilepsy: Practical Implementation of Ketogenic Therapy, First Edition. John Wiley & Sons, Ltd, 2012:166-171.

28. Smith EH MD. Acylcarnitine Analysis by Tandem Mass Spectrometry. Curr Protoc Hum Genet 2010;64:17.8.1,17.8.20.

29. Ringseis R, Keller J, Eder K. Role of carnitine in the regulation of glucose homeostasis and insulin sensitivity: evidence from in vivo and in vitro studies with carnitine supplementation and carnitine deficiency. Eur J Nutr 2012;51:1-18.

30. Rebouche CJ CC. Metabolic fate of dietary carnitine in human adults: identification and quantification of urinary and feed metabolites. J Nutr 1991;121:S39-46.

31. Reuter SE EA. Carnitine and Acylcarnitines: Pharmacokinetic, Pharmacological and Clinical Aspects. Clin Pharmacokinet 2012;51(9):553-72.

32. Lombard KA, Olson AL, Nelson SE, et al. Carnitine status of lactoovovegetarians and strict vegetarian adults and children. Am J Clin Nutr 1989;50(2):301-6.

33. Stadler DD, Chenard CA, Rebouche CJ. Effect of dietary macronutrient content on carnitine excretion and efficiency of carnitine reabsorption. Am J Clin Nutr 1993;58(6):868-72.

34. American Academy of Pediatrics, Committee on Nutrition. Soy protein-based formulas: recommendations for use in infant feeding. Pediatrics 1998;101(1):148-52.

35. Sandor A, Pecsuvac K, Kerner J, et al. On carnitine content of the human breast milk. Pediatr Res 1982;16(2):89-91.

36. Berry-Kravis E, Booth G, Sanchez AC. Carnitine levels and the ketogenic diet. Epilepsia 2001;42:1445-51.

37. De Vivo DC, Tein I. Primary and secondary disorders of carnitine metabolism. Int Pediatr 1990;5:134-41.

38. Scaglia F, Wang Y, Singh RH, et al. Defective urinary carnitine transport in heterozygotes for primary carnitine deficiency. Genet Med 1998;1:34-9.

39. Johnson DW. An acid hydrolysis method for quantification of plasma free and total carnitine by flow injection tandem mass spectrometry. Clin Biochem 2010;43:1362-7.

40. Khan L, Bamji MS. Plasma carnitine levels in children with protein-calorie malnutrition before and after rehabilitation. Clinica Chimica Acta 1977;75:163-6.

41. Alp H, Orbak Z, Akcay F, et al. Plasma and urine carnitine levels and carnitine supplementation in children with malnutrition. J Trop Pediatr 1999;45:294-6.

42. Hartman AL, Gasior M, Vining EPG. The Neuropharmacology of the Ketogenic Diet. Pediatr Neurol 2007;36(5):281-92.

43. Pan JW, de Graaf RA, Petersen KF, Shulman GI, et al. [2,4-13C2]-β- Hydroxybutyrate metabolism in human brain. J Cereb Blood Flow Metab 2002;22:890-8.

44. Pierre K PL. Monocarboxylate transporters in the central nervous system: distribution, regulation and function. J Neurochem 2005;94:1-14.

45. Bieber LL, Emaus R, Valkner K. Possible functions of short-chain and medium-chain carnitine acyltransferases. Fed Proc 1982;41(12):2858-62.

46. Rebouche CJ EA. Kinetic compartmental analysis of carnitine metabolism in the human carnitine deficiency syndromes. Evidence for alterations in tissue carnitine transport. J Clin Invest 1984;73(3):857-67.

47. Peng M, Liu L, Jiang M, Liang C, Zhao X, Cai Y, Sheng H, Ou Z, Luo H. Measurement of free carnitine and acylcarnitines in plasma by HILIC-ESI-MS/MS without derivatization. Journal of Chromatography B 2013;932:12-8.

48. Chacea DH, Kalas TA, Naylor EW. Use of Tandem Mass Spectrometry for Multianalyte Screening of Dried Blood Specimens from Newborns. Clin Chem 2003;49(11):1797-817.

49. Stevens RD, Hillman SL, Worthy S. Assay for Free and Total Carnitine in Human Plasma Using Tandem Mass Spectrometry. Clin Chem 2000;46(5):727-9.

50. ARUP Laboratories. Carnitine Panel. Version current 2014. Internet: http://ltd.aruplab.com/Tests/Pub/0081110 (accessed September 3 2014).

51. Costa CC, De Almeida IT, Jakobs C, et al. Dynamic Changes of Plasma Acylcarnitine Levels Induced by Fasting and Sunflower Oil Challenge Test in Children. Pediatr Res 1999;46:440-50.

52. Engel AG, Rebouche Cl, Wilson DM, et al. Primary systemic carnitine deficiency. II. Renal handling of carnitine. Neurology 1981;31:819-25.

53. Coppola G, Epifanio G, Auricchio G, Federico R, Resicato G, Pascotto A. Plasma free carnitine in epilepsy children, adolescents and young adults treated with old and new antiepileptic drugs with or without ketogenic diet. Brain Dev 2006;28:358-65.

54. Stadler DD, Bale JF, Chenard CA, Rebouche CJ. Effect of long-term valproic acid administration on the efficiency of carnitine reabsorption in humans. FASEB J 1996;10A:470.

55. ARUP Laboratories. Acylcarnitine Quantitative Profile, Plasma. Version current 2014. Internet: http://ltd.aruplab.com/Tests/Pub/0040033 (accessed September 3 2014).

56. Coulter DL. Carnitine deficiency in epilepsy: Risk factors and treatment. J Child Neurol 1995;10(2):32-9.

57. Sahajwalla CG, Helton ED, Purich ED, et al. Multiple-dose pharmacokinetics and bioequivalence of L-carnitine 330-mg tablet versus 1-g chewable tablet versus enteral solution in healthy adult male volunteers. J Pharm Sci 1995;84(5):627-33.

58. Koeth R, Wang Z, Levison B. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 2013;19:

59. Zeger SL,Liang K, Albert PS. Models for Longitudinal Data: A Generalized Estimating Equation Approach. Biometrics 1988;44(4):1049-60.

60. Rigault C, Le Borgne F, Tazir B, et al. A high-fat diet increases L-carnitine synthesis through a differential maturation of the Bbox1 mRNAs. Biochem Biophys Acta 2013;1831(2):370-7.

61. Kossoff EH, McGrogan JR, Freeman JM. Benefits of an All-Liquid Ketogenic Diet. Epilepsia 2004;45(9):1163-.

62. Abbott Nutrition. RCF® No Added Carbohydrate Soy Infant Formula Base With Iron. Internet: http://abbottnutrition.com/brands/products/rcf (accessed 05/05 2015).

63. Nutricia. KetoCal 4:1 LQ. Internet: http://www.myketocal.com/ketocal4-1.aspx (accessed 5/3 2015).

64. Nestle Health Science. Nutren Junior Fiber. Internet: http://www.nestlenutritionstore.com/products/Pediatric-Nutrition/Nutren-Junior-Fiber.html#.VVGgFutZVUQ (accessed May 11 2015).

65. Rebouche CJ, Bosch EP, Chenard CA, et al. Utilization of dietary precursors for carnitine biosynthesis in human adults. J Nutr 1989;119:1907-13.

66. Smith EH, Matern D. Acylcarnitine Analysis by Tandem Mass Spectrometry. In: Current Protocols in Human Genetics. John Wiley & Sons, Inc., 2001:

67. Kien CL, Everingham KI, Stevens RD, et al. Short-Term Effects of Dietary Fatty acids on Muscle Lipid Composition and Serum acylcarnitine Profile in Human Subjects. Obesity 2011;19:305-11.

68. Koves TR, Ussher JR, Noland RC, et al. Mitochondrial Overload and Incomplete Fatty Acid Oxidation Contribute to Skeletal Muscle Insulin Resistance. Cell Metab 2008;7(1):45-56.

69. Power GW, Cake MH, Newsholme EA. Influence of Diet on the Kinetic Behavior of Hepatic Carnitine Palmitoyltransferase I Toward Different Acyl CoA Esters. Lipids 1997;32(1):31-7.

70. United States Department of Agriculture Agricultural Research Service. National Nutrient Database for Standard Reference Release 27. Internet: http://ndb.nal.usda.gov/ndb/nutrients/index (accessed 5/3 2015).

71. Engel AG, Rebouche CJ, Wilson DM, et al. Primary systemic carnitine deficiency: II. renal handling of carnitine. Neurology 1981;31:819-25.

72. Newgard CB, An J, Bain JR. A Branched-Chain Amino Acid-Related Metabolic Signature that Differentiates Obese and Lean Humans and Contributes to Insulin Resistance. Cell Metabolism 2009;9:311-26.

73. Brosnan JT BM. Branched-Chain Amino Acids: Enzyme and Substrate Regulation. J Nutr 2006;136(1):207S-11S.

74. Evangeliou A, Spilioti M, Doulioglou V. Branched Chain Amino Acids as Adjunctive Therapy to Ketogenic Diet in Epilepsy: Pilot Study and Hypothesis. J Child Neurol 2009;24:1268-72.

APPENDIX

Appendix A

Research consent and authorization form

IRB#: IRB00010679

Research Consent Summary

You are being asked to join a research study. You do not have to join the study. Even if you decide to join now, you can change your mind later.

1. The purpose of this study is to learn more about how the ketogenic diet, which is used to treat seizure disorders in children, affects the amount of carnitine in the blood. Carnitine is a vitamin-like substance found normally in blood and muscle tissue. Carnitine is used by our bodies to breakdown fat and to create ketones. Ketones are the substances formed when a ketogenic diet is eaten and they may reduce the number or severity of seizures in children with epilepsy.
2. We want to learn
    a. if consuming a ketogenic diet causes the amount of carnitine in the blood to decrease in children with epilepsy.
3. The Academy of Nutrition and Dietetics Foundation and the Academy of Nutrition and Dietetics Pediatric Nutrition Practice Group is paying for this research study.
4. If you choose to participate in this study, information will be obtained from your medical record, including information about past and present medicine use, height and weight, ketogenic diet prescription, blood sample results, and medical history. All of this information will be recorded anonymously. Only information collected as part of your routine care will be recorded including information before starting the ketogenic diet though 6 months of treatment. No information will be collected unless you decide to participate in this study. You may decide to stop treatment with the ketogenic diet at any time and this decision will not affect your participation in this study. You will not be individually identified in any research reports, and all information about you will be kept completely confidential. There will be no added costs associated with participating in this study and there will be no follow up after this study is completed.

OREGON HEALTH & SCIENCE UNIVERSITY

IRB#: IRB00010679

Research Consent and Authorization Form

TITLE: Carnitine status of children treated with the ketogenic diet for intractable epilepsy

PRINCIPAL INVESTIGATOR: Dr. Diane Stadler, PhD, RD (503) 494-0168

CO-INVESTIGATORS: Dr. Carter Wray, MD (503) 494-9113
Dr. Melanie Gillingham, PhD, RD (503) 494-1682
Jane Riebold, BS (503) 494-1309

FUNDED BY: Pediatric Nutrition Practice Group Research Grant Award (Academy of Nutrition and Dietetics) and Herbert D. and Nyelda Gemple Research Award (Academy of Nutrition and Dietetics)

PURPOSE:
"You" means you or your child throughout this form.

You have been invited to be in this research study because you are being treated by the ketogenic diet for epilepsy. The purpose of this study is to learn how the ketogenic diet, which is used to treat seizure disorders in children, affects the amount of carnitine in the blood.

Carnitine is a vitamin-like substance found normally in blood and muscle tissue. Carnitine is used by our bodies to break down fat and to create ketones. Ketones are the substances formed when a ketogenic diet is eaten and they may reduce the number or severity of seizures in children with epilepsy. We want to learn if consuming a ketogenic diet causes the amount of carnitine in the blood to decrease in children with epilepsy.

We hope to include as many as 25 subjects in this study at OHSU.

PROCEDURES:
If you choose to participate in this study, information will be obtained from your medical record for research purposes.

When possible, information will be collected before you begin the ketogenic diet and through 6 months of treatment. This information will be recorded in your medical record regardless of whether or not you choose to participate in this study, but will only be used for research purposes if you do decide to participate.

We will collect information from your medical record for this study, including your medical history, past and present medicine use, height and weight, ketogenic diet prescription, and blood sample results.

If you have any questions, concerns, or complaints regarding this study now or in the future, contact Dr. Diane Stadler, PhD, RD, at (503) 494-0168 or other members of the study team at (503) 494-1309.

RISKS AND DISCOMFORTS:
Although we will take steps to protect your identity, there is a risk of breach of confidentiality.

BENEFITS:
You will not benefit from being in this study. However, by serving as a subject, you may help us learn how to benefit patients in the future.

ALTERNATIVES:
You may choose not to be in this study.

CONFIDENTIALITY:
We will take steps to keep your information confidential, but we cannot guarantee total privacy. All data collected by medical record review will be coded with a random number identifier to protect confidentiality.

We will collect health information about you as described in the Purpose and Procedures sections of this form. Health information is private and is protected under federal law and Oregon law. By agreeing to be in this study, you are giving permission (also called authorization) for us to use and disclose your health information as described in this form.

The investigators, study staff, and others at OHSU may use the information we collect and create about you to conduct and oversee this research study.

We may release this information to others outside of OHSU who are involved in conducting or overseeing research, including the Office for Human Research Protections, a federal agency that oversees research involving humans, who may be permitted to review and copy your records, including your medical records.

We will not release information about you unless required or permitted by law. We will not use your name or your identity for publication or publicity purposes, unless we have your special permission.

When we send information outside of OHSU, it may no longer be protected under federal or Oregon law. In this case, your information could be used and re-released without your permission.

We may continue to use and disclose your information as described above indefinitely.

The information collected and created in this study will not be placed in your OHSU medical record. If you have questions about what study information you will be able to access, and when, ask the investigator.

COMMERCIAL DEVELOPMENT:
Information about you or obtained from you in this research may be used for commercial purposes, such as making a discovery that could, in the future, be patented or licensed to a company, which could result in a possible financial benefit to that company, OHSU, and its researchers. There are no plans to pay you if this happens. You will not have any property rights or ownership or financial interest in or arising from products or data that may result from your participation in this study. Further, you will have no responsibility or liability for any use that may be made of your information.

COSTS:
There will be no cost to you or your insurance company to participate in this study.

LIABILITY:
If you believe you have been injured or harmed as a result of participating in this research and require treatment, contact Dr. Diane Stadler, PhD, RD at (503) 494-0168.

If you are injured or harmed by the study procedures, you will be treated. OHSU and the Academy of Nutrition and Dietetics Foundation do not offer any financial compensation or payment for the cost of treatment if you are injured or harmed as a result of participating in this research. Therefore, any medical treatment you need may be billed to you or your insurance. However, you are not prevented from seeking to collect compensation for injury related to negligence on the part of those involved in the research. Oregon law (Oregon Tort Claims Act (ORS 30.260 through 30.300)) may limit the dollar amount that you may recover from OHSU or its caregivers and researchers for a claim relating to care or research at OHSU, and the time you have to bring a claim.

If you have questions on this subject, please call the OHSU Research Integrity Office at (503) 494-7887.

PARTICIPATION:
This research is being overseen by an Institutional Review Board ("IRB"). You may talk to the IRB at (503) 494-7887 or irb@ohsu.edu if:

- Your questions, concerns, or complaints are not being answered by the research team.
- You want to talk to someone besides the research team.
- You have questions about your rights as a research subject.
- You want to get more information or provide input about this research.

You may also submit a report to the OHSU Integrity Hotline online at https://secure.ethicspoint.com/domain/media/en/gui/18915/index.html or by calling toll-free (877) 733-8313 (anonymous and available 24 hours a day, 7 days a week).

You do not have to join this or any research study. You do not have to allow the use and disclosure of your health information in the study, but if you do not, you cannot be in the study.

If you do join the study and later change your mind, you have the right to quit at any time. This includes the right to withdraw your authorization to use and disclose your health information. If you choose not to join any or all parts of this study, or if you withdraw early from any or all parts of the study, there will be no penalty or loss of benefits to which you are otherwise entitled, including being able to receive health care services or insurance coverage for services. Talk to the investigator if you want to withdraw from the study.

If you no longer want your health information to be used and disclosed as described in this form, you must send a written request or email stating that you are revoking your authorization to:

Dr. Diane Stadler, PhD, RD
Oregon Health & Science University
3181 SW Sam Jackson Park Road
Mail code: GH207
Portland, Oregon 97239

Email: stadler@ohsu.edu

Your request will be effective as of the date we receive it. However, health information collected before your request is received may continue to be used and disclosed to the extent that we have already acted based on your authorization.

If in the future you decide you no longer want to participate in this research, we will remove your name and any other identifiers from your samples and information, but the material will not be destroyed and we will continue to use it for research.

You may be removed from the study if you are unable to continue the ketogenic diet for at least six months, are not able to regularly attend scheduled clinic visits, or if you receive supplemental carnitine as part of treatment with the ketogenic diet.

We will give you any new information during the course of this research study that might change the way you feel about being in the study.

Your health care provider may be one of the investigators of this research study and, as an investigator, is interested in both your clinical welfare and in the conduct of this study. Before entering this study or at any time during the research, you may ask for a second opinion about your care from another doctor who is in no way involved in this project. You do not have to be in any research study offered by your physician.

SIGNATURES:

Your signature below indicates that you have read this entire form and that you (or as a representative of your child) agree to be in this study.

We will give you a copy of this signed form.

OREGON HEALTH & SCIENCE UNIVERSITY

INSTITUTIONAL REVIEW BOARD

PHONE NUMBER (503) 494-7887

CONSENT/AUTHORIZATION FORM APPROVAL DATE

Dec. 24, 2014

Do not sign this form after the expiration date of: 11/15/2015

Subject Printed Name	Subject Signature (if applicable)	Date
Subject Parent or Guardian Printed Name	Subject Parent or Guardian Signature	Date

Relationship of Parent or Guardian to Subject

Complete if the participant is not fluent in English and an interpreter was used to obtain consent. Participants who do not read or understand English must not sign this full consent form, but instead sign the short form translated into their native language. This form should be signed by the investigator and interpreter only.

Print name of interpreter: _____

Signature of interpreter: _____ Date: _____

An oral translation of this document was administered to the subject in _____ (state language) by an individual proficient in English and _____ (state language).

See the attached short form for documentation.

Appendix B

Research child assent form

Child Assent Form

IRB#: IRB00010679

TITLE: Carnitine status of children treated with the ketogenic diet for intractable epilepsy.

PRINCIPAL INVESTIGATOR: Dr. Diane Stadler, PhD, RD (503) 494-0168

CO-INVESTIGATORS: Dr. Carter Wray, MD (503) 494-9113
Dr. Melanie Gillingham, PhD, RD (503) 494-1682
Jane Riebold, BS (503) 494-1309

This research study was explained to me. I know how it may or may not help me. I also know that this study will help doctors learn more about treating children with seizure disorders. To be sure that I know what is going to happen, the investigator will ask me the following:

1. To explain what I will do and what will happen in this study.

2. If I have any questions or want to know anything else about this study or about using the ketogenic diet to treat seizure disorder.

3. To explain some of the good and bad things that might happen to me if I enter this study.

I have thought about being a part of this study. I have asked and received answers to my questions. I agree to be in this study. I know that I don't have to agree to be in the study. Even though I agree to be in it now, I know I may feel differently later on and can ask to stop being in the study. I know that I may talk with my parents and/or doctor about not being in this study at any time.

OREGON HEALTH & SCIENCE UNIVERSITY

INSTITUTIONAL REVIEW BOARD

PHONE NUMBER (503) 494-7887

CONSENT/AUTHORIZATION FORM APPROVAL DATE

Dec. 24, 2014

Do not sign this form after the expiration date of: 11/15/2015

Name/signature:_____ Date:_____

Appendix C

List of Doernbecher Children's Hospital ketogenic diet laboratory protocols

Pre-Ketogenic labs, if they have not been already obtained during work up

- ❑ CBC, Differential
- ❑ Acylcarnitine, profile, PLASMA
- ❑ Complete Metabolic Set (Na, K, Cl, CO2, BUN, Creat., Glucose, Ca, AST, ALT, Bili total, Alk phos, Alb, Prot total)
- ❑ Phosphorus
- ❑ Magnesium
- ❑ Serum amino acids
- ❑ FASTING Lipid Profile (LIPID SET to include Triglycerides & Cholesterol)
- ❑ Zinc, serum
- ❑ Selenium, serum
- ❑ Vitamin D, 25- Hydroxy, serum
- ❑ Urine organic acids
- ❑ Urine calcium
- ❑ Urine creatinine
- ❑ PTH, serum
- ❑ Uric acid, Serum
- ❑ β-hydroxybutyric acid, PLASMA
- ❑ Ketone body screen, plasma also called Acetoacetate
- ❑ Lab other "Carnitine, Free & Total (Includes Carnitine, Esterified)" ARUP 0080068, 1 ml in green or red top

Follow-up Ketogenic labs at 1 month

- ❑ β-hydroxybutyric acid, PLASMA
- ❑ Ketone body screen, plasma also called Acetoacetate
- ❑ Basic Metabolic Set (Na, K, Cl, CO2, BUN, Creat., Gluc, Ca)
- ❑ Urinalysis
- ❑ Lab other "Carnitine, Free & Total"

Follow-up Ketogenic labs at 3 and 9 months

- ❑ β-hydroxybutyric acid, PLASMA
- ❑ Ketone body screen, plasma also called Acetoacetate
- ❑ CBC, Differential

- ❑ Complete Metabolic Set (Na, K, Cl, CO2, BUN, Creatinine, Glucose, Ca, AST, ALT, Bili total, Alk phos, Alb, Prot total)
- ❑ FASTING Lipid Profile (LIPID SET)
- ❑ Urinalysis
- ❑ Lab other "Carnitine, Free & Total"

Follow-up Ketogenic labs at 6 and 12 months

- ❑ β-hydroxybutyric acid, PLASMA
- ❑ Ketone body screen, plasma also called Acetoacetate
- ❑ CBC, Differential
- ❑ Complete Metabolic Set (Na, K, Cl, CO2, BUN, Creatinine, Glucose, Ca, AST, ALT, Bili total, Alk phos, Alb, Prot total)
- ❑ FASTING Lipid Profile (LIPID SET)
- ❑ Urinalysis
- ❑ Acylcarnitine, profile, PLASMA
- ❑ Zinc, serum
- ❑ Selenium, serum
- ❑ Vitamin D, 25-Hydroxy, serum
- ❑ Phosphorus
- ❑ Magnesium
- ❑ Urine calcium
- ❑ Urine creatinine
- ❑ Lab other "Carnitine, Free & Total"

If concern for kidney stones:

- ❑ Basic Metabolic Set (Na, K, Cl, CO2, BUN, Creat., Gluc, Ca)
- ❑ Ionized calcium
- ❑ Urinalysis with microscopy
- ❑ Urine calcium
- ❑ Urine creatinine
- ❑ PTH, serum
- ❑ Uric acid, Serum
- ❑ Urinalysis

If Anemic:

- ❑ Ferritin
- ❑ Total Iron
- ❑ Reticulocyte count

Appendix D

Evidence analysis table

Study identification	Participants	Design	Duration	Intervention	Outcomes
Freeman JM, Vining EP, Kossoff EH, et al. A blinded, crossover study of the efficacy of the ketogenic diet. Epilepsia 2009;50:322-5.	• 20 children with intractable Lennox-Gastaut syndrome • Mean age of 3.9 years (1.0 – 7.4) • 11 male and 9 female	Single-blinded randomized crossover study	12 days with an additional three days before intervention and at the midpoint of the study	• Participants enrolled 1997 – 2002 • Fasted 36 hours then assigned to a classic ketogenic diet with additional 60 g/day of glucose/saccharin (11 given glucose first) • 4:1 ketogenic ratio (n=13, 65%) or 3:1 ketogenic ratio (n=7) • Crossover to the alternate solution occurred after day 6 and a repeat fast	• Reduction in parent-reported seizures between the glucose and saccharin arms • Median difference 1.5 seizures/day (p=0.07) • No reduction in EEG-identified events • Median reduction of 7 events/day (p=0.33) • Ketosis not completely eliminated in the glucose-added arm
Neal EG, Chaffe H, Schwartz RH, et al. The ketogenic diet for the treatment of childhood epilepsy: a randomised controlled trial. Lancet Neurol 2008;7:500-6.	• 145 children • Age 2 – 16 years • Daily seizures or >7 per week • Failed to respond to at least 2 AEDs • Not treated previously with the KD	Randomized controlled trial	3 months, with enrollment from December 2001 and July 2006	• Children were randomly assigned to receive a ketogenic diet, either immediately (73) or after a 3-month delay (72, control group) • Early withdrawals were recorded, and seizure frequency on the diet was assessed after 3 months and compared with that of the controls • Tolerability of the diet was assessed by questionnaire at 3 months	• Mean percentage of baseline seizures was significantly lower in the diet group than the controls (62.0% vs. 136.9%, 95% CI 42.4 – 107.4%; p<0.01) • 28 children (38%) in the diet group had >50% seizure reduction compared with 4 (6%) controls (p<0.01), and 5 children (7%) in the diet group had >90% seizure reduction (p=0.06) • No significant difference in treatment efficacy between syndromes
Berry-Kravis E, Booth G, Sanchez AC. Carnitine levels and the ketogenic diet. Epilepsia 2001;42:1445-51.	• 38 consecutive patients who initiated the KD from May 1997 to March 2000 • 8 patients who started on the diet before May 1997	Unblinded uncontrolled study	24 months	• Carnitine concentration at 0, 1, 6, 12, and 24 months of diet treatment, carnitine antiepileptic drug (AED) history, lowest blood glucose and time to achieve ketosis during diet initiation, and diet complications were analyzed • Carnitine concentration at follow-up were analyzed for eight patients who initiated diet before to May 1997	• Multiple AED exposure lowered TC • TC deficiency was not common and occurred without clinical symptoms • TC levels did not predict hypoglycemia or problems achieving ketosis • TC stabilized or increased back toward baseline with long-term treatment • Most patients did not require carnitine supplementation

Coppola G, Epifanio G, Auricchio G, et al. Plasma free carnitine in epilepsy children, adolescents and young adults treated with old and new antiepileptic drugs with or without ketogenic diet. Brain Dev 2006;28:358-65.	• 164 epilepsy patients • Age 7 months – 30 years (mean 10.8 years) • Treated for a mean of 7.5 years (1 month – 26 years) with antiepileptic drugs as mono or add-on therapy	Cross-sectional study with a clinical trial in one subset; enrollment ran from January 2002 to June 2003	12 months	• Free carnitine levels were evaluated for all patients • A subset of patients was administered ketogenic diet in addition to their baseline AEDs • In 16 patients on topiramate or lamotrigine and in 11 on ketogenic diet, plasma free carnitine was prospectively evaluated before starting treatment and after 3 and 12 months, respectively	• Low plasma levels of free carnitine were found in 41 patients (25%) • 32 out of 84 patients (38%) taking valproic acid and 13 of 54 (24%) on carbamazepine showed low free carnitine levels • Valproic acid was associated with a higher risk of hypocarnitinemia (27.3%) compared carbamazepine (14.3%) • No patients on topiramate (10), lamotrigine (5) or ketogenic diet (11) developed hypocarnitinemia during the first 12 months of treatment
Koeth R, Wang Z, Levison B. Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis. Nat Med 2013;19.	X	Cross-sectional studies, epidemiological studies, in vivo tissue studies, and controlled unblinded research study	X	X	• Metabolism by intestinal microbiota of dietary L-carnitine, also produces TMAO and accelerates atherosclerosis in mice • Omnivorous human subjects produced more TMAO than did vegans or vegetarians following ingestion of L-carnitine through a microbiota-dependent mechanism • The presence of specific bacterial taxa in human feces was associated with plasma TMAO concentration and dietary status • Plasma L-carnitine levels in subjects undergoing cardiac evaluation (n = 2,595) predicted increased risks for both prevalent cardiovascular disease (CVD) and incident major adverse cardiac events (myocardial infarction, stroke or death), but only among subjects with high TMAO levels • Chronic dietary L-carnitine supplementation in mice altered cecal microbial composition, markedly enhanced synthesis of TMA and TMAO, and increased atherosclerosis, but this

(Koeth, et al., continued)					did not occur if intestinal microbiota was concurrently suppressed • In mice with an intact intestinal microbiota, dietary supplementation with TMAO or either carnitine or choline reduced in vivo reverse cholesterol transport • Intestinal microbiota may thus contribute to the well-established link between high levels of red meat consumption and CVD risk
Hug G, McGraw CA, Bates SR. Reduction of serum carnitine concentrations during anticonvulsant therapy with phenobarbital, valproic acid, phenytoin, and carbamazepine in children. J Pediatr 1991;119:799-802.	• 471 patients treated for convulsions with phenobarbital, valproic acid, phenytoin, and/or carbamazepine • 8 treatment groups; four treated with monotherapy and four with polytherapy	Observational study	X	• Analyzed four carnitine constituents in serum of participants (total and free carnitine and short- and long-chain fatty acid carnitine esters)	• Means of all four carnitine constituents were significantly reduced in all groups (except for free carnitine in four groups) • Total carnitine was reduced by 23% to 48%, free carnitine by 9% to 45%, short-chain fatty acid carnitine by 46% to 64%, and long-chain fatty acid carnitine by 6% to 29% • Patient frequency of reduction for total carnitine was 20% of all patients (10% for free carnitine), 23% of patients receiving valproate (9% for free carnitine), 36% of those receiving phenobarbital (21% for free carnitine), 12% of those receiving phenytoin (8% for free carnitine), and 8% of those receiving carbamazepine (1% for free carnitine)
Likhodii SS BW. Ketogenic diet: does acetone stop seizures? Med Sci Monit 2002;8:19-24.	Rats	Unblinded controlled study	10 days	• Rats were injected with 1 or 10 mmol/kg acetone or received acetone in drinking water (1% v/v) for 10 days and were then injected with 1 mmol/kg dose of acetone • Pentylenetetrazole seizure test was administered 15 min after injections followed by measurement of acetone in the cerebrospinal fluid	• A 10 mmol/kg injection of acetone suppressed seizures in 60% of rats • Chronic administration of acetone followed by a 1 mmol/kg injection suppressed seizures in 47% of rats • 1 mmol/kg injection (without acetone pretreatment) did not have a statistically significant effect

Hemingway C, Freeman JM, Pillas DJ. The ketogenic diet: a 3- to 6-year follow-up of 150 children enrolled prospectively. Pediatrics 2001;108:898-905.	150 children with difficult-to-control seizures	Long-term observational study	3 to 6 years	• Three to 6 years after diet initiation, 150 families were sent a survey • Inquiring about their child's current health status, seizure frequency, and current anticonvulsant medications as well as their experience with the diet and reasons for discontinuation	• Of the original 150 patient cohort, 20 (13%) were seizure-free and an additional 21 (14%) had a 90% to 99% decrease in their seizures • Twenty-nine were free of medications, and 28 were on only 1 medication • 15 remained on the diet • There were no known cardiac complications