OREGON HEALTH AND SCIENCE UNIVERSITY

Dietary Intake and Plasma Acylcarnitine among LCHADD/TFPD Participants

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Specific Aims

Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) and Trifunctional Protein Deficiency (TFPD) are rare long-chain fatty acid oxidation disorders (LC-FAOD) associated with high morbidity and mortality rates. Infants that present with the severe phenotype often experience symptoms such as hypoglycemia, hepatomegaly, encephalopathy, and cardiomyopathy soon after birth. Intermediate and mildly affected infants may experience hypoketotic hypoglycemia, myopathy, and/or neuropathy.[1] While these complications can be fatal, early detection and initiation of medical nutrition therapy have been shown to significantly increase infant survival.[2] Chronic complications persist despite diagnosis and include peripheral neuropathy, retinopathy, and recurring rhabdomyolysis.[1] Identification of newborns is accomplished via screening for the presence of long-chain hydroxyacylcarnitines (OH-ACs) in dried blood spots during routine newborn screening (NBS). Infants with elevated OH-ACs are subsequently evaluated with diagnostic tests which can include additional plasma acylcarnitines or molecular analysis. Elevated long-chain OH-ACs are pathoneumonic for LCHADD/TFPD.[3]

Medical nutrition therapy (MNT) recommendations for patients with LCHADD includes avoiding excessive fasting and preventing long-chain fatty acids from being used for energy with small frequent meals. Current recommendations are based on relatively few studies and are primarily derived from case reports and basic knowledge of the disorder. Most nutrition recommendations suggest limiting long-chain fatty acid intake because patients with LCHADD/TFPD are unable to oxidize these fats. In addition, patients are often counseled to supplement their diet with medium-chain triglycerides (MCT) for which the medium-chain fatty acid oxidation pathway remains intact. Limiting dietary fat necessitates increasing calories from either protein, carbohydrate or MCT sources.[4] MCT oil is predominately C8, C10, and C12 fatty

acids and is the most common fatty acid supplement used in this population to replace long-chain fat. However, triheptanoin (Dojolvi®), an odd-chain seven-carbon triglyceride, is an effective alternative with cardiac benefits that was recently approved by the FDA for treatment of LC-FAODs.[5]

Following a low-fat diet supplemented with MCT has been associated with lower plasma hydroxyacylcarnitines in LCHADD/TFPD.[6] However, evidence linking lower plasma acylcarnitine profiles with improved outcomes such as preventing recurrent rhabdomyolysis or cardiomyopathy is lacking. There is one publication that reported a correlation between lower plasma OH-ACs and better retinal function among patients with LCHAD.[7] Our current MNT recommendations of small frequent meals certainly prevents episodes of hypoglycemia in young patients. It may improve biochemical markers of the disease – i.e., lower OH-ACs, but evidence for the long-term benefit of this diet is very limited. In addition, long-chain fatty acids are the primary source of fatty acids in the majority of food sources. Because the current MNT recommends a very low-fat diet, following these recommendations can be challenging for individuals with this condition. More data is needed to better establish the relationship between dietary intake, plasma ACs, and signs and symptoms of the disease such as recurrent rhabdomyolysis, cardiomyopathy, peripheral neuropathy and chorioretinopathy.

Understanding how patients and families with LCHADD are managing current dietary recommendations is critical for establishing the efficacy of our current MNT. An intake of 10-20% of dietary long-chain fat is recommended for these patients[2], but there is little research exploring compliance with this recommendation across a wide range of ages. Likewise, there is limited data on the relationship between dietary fat intake and plasma hydroxy-acylcarnitine

concentrations in plasma. It is currently unknown if patients who follow MNT guidelines have lower plasma OH-ACs and/or improved long-term outcomes.

The goal of this project is to describe the usual intake of these patients across their lifespan and determine the correlation between their dietary fat intake on hydroxy-acylcarnitine concentrations. This project will help fill the gaps in knowledge surrounding the current real-world implementation of MNT among patients with LCHADD/TFPD. We will compare three-day diet records in a cohort of subjects with a wide age range; the youngest participant is 2 years old and the oldest at 36 years old. The total cohort of 40 participants with LCHADD/TFPD were recruited to participate in the Natural History of LCHADD retinopathy study.

The specific aims of this project are:

Specific Aim 1: To describe the macro- and micronutrient composition of usual intake in LCHADD/TFPD patients aged 2-36 yr using three-day diet records, including any supplements consumed.

Hypothesis: Younger subjects will consume fewer long-chain fats and more medium-chain fatty acids than older subjects.

Specific Aim 2: To correlate dietary long-chain and medium-chain fat intake with fasting plasma acylcarnitine levels.

Hypothesis: A diet low in long-chain fatty acids supplemented by MCT oil will correlate with lower plasma acylcarnitine levels.

Current MNT recommendations for LCHADD/TFPD are based on case studies and very small cohort studies of about 11 participants. This current analysis will examine dietary intake in a large cohort of participants with a relatively large age range to evaluate how patients and

families are currently implementing dietary recommendations for managing this rare genetic disease.

Background

Introduction

Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) and Trifunctional Protein Deficiency (TFPD) are both long-chain fatty acid oxidation disorders (LC-FAOD). Occurring between 1/75,000 and 1/250,000 births in the United States, these rare, autosomal recessive genetic disorders are associated with high morbidity and mortality rates.[8] The ability to screen infants for LC-FAODs by acylcarnitine analysis of dried blood spots was developed in the 1990s. Expanded newborn screening (NBS) by tandem mass spectroscopy (MS/MS) was implemented in the early 2000s and included screening for LCHADD/TFPD. The early screening and prevention provided by NBS have led to significantly reduced mortality rates of individuals affected. Although they are distinct disorders, LCHADD and TFPD are both due to genetic variants and loss of function within the same protein complex, mitochondrial trifunctional protein (TFP).

Pathophysiology

The mitochondrial trifunctional protein (TFP) is one of the proteins needed for proper long-chain fat metabolism. TFP is a complex of 2 α- and 2 β-subunits that are active in the last three steps of β-oxidation.[9-10] The enzymatic activities for these steps are long-chain 3- enoyl-CoA hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), and long-chain keto-thiolase. The *HADHA* gene encodes the α-subunit of the TFP which catalyzes both long-chain enoyl-CoA hydratase and LCHAD activities. In contrast, the *HADHB* gene encodes the β-subunit that catalyze the long-chain thiolase activity.[8] TFPD occurs when mutations are present in either the *HADHA* or *HADHB* genes that result in the loss of function of all three enzymatic processes. However, the more common missense variant, c. 1528G>C, results in the change of one amino

acid in the LCHADD binding domain. There is a glutamic acid to glutamine amino acid change (E510Q) in the LCHAD active site of TFPa reducing its activity but leaving the other two enzymatic activities relatively intact. Thus, patients with one or two copies of c.G1528C have LCHAD deficiency.[8,11]

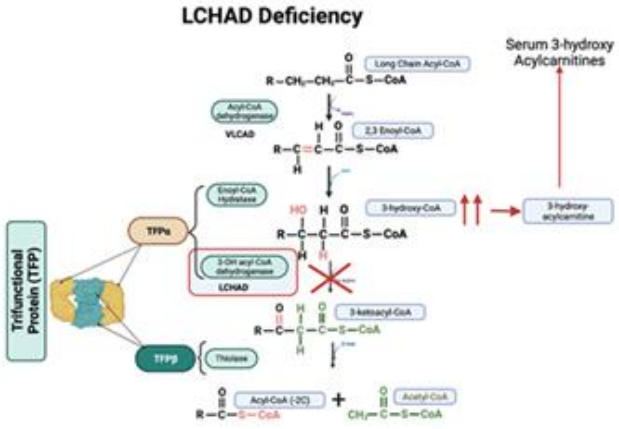
In normal fatty acid oxidation, long-chain fatty acids enter the mitochondria through the carnitine shuttle. Before fatty acids can move through the carnitine shuttle the fatty acid is esterified to coenzyme A and the fatty acyl-CoA is converted to acylcarnitine by carnitine palmitolyltransferase 1 (CPT1). The long-chain acylcarnitine is transported into the mitochondria by carnitine acylcarnitine transferase (CACT). Then, the fatty acylcarnitine is reconverted to a long-chain acyl-CoA by carnitine palmiotyltransferase 2 (CPT2). In the first step in fatty acid oxidation, very long-chain acyl-CoA dehydrogenase (VLCAD) creates a double bond between carbons 2 and 3 of the fatty acid. The hydrogens are transferred to the ETF of the respiratory chain to create energy. The next 3 enzymatic steps of TFP shorten the fatty acid by 2 carbons, creating one acetyl-CoA that enters the TCA cycle and one chain-shortened fatty acid to continue with oxidation. This process is disturbed in patients with LCHADD and TFPD.

Among patients with LCHAD deficiency carrying the common c. 1528G>C mutation the upstream long-chain hydratase creates a hydroxyl group on the 3rd carbon using water. The subsequent LCHAD activity is dramatically decreased (about 10% of normal). The block in LCHAD activity leads to the accumulation of the 3-hydroxy fatty acylCoA in the mitochondria. Among patients with TFP deficiency, there is a decrease of long-chain hydratase along with LCHADD and thiolase so fewer 3-hydroxy acylCoAs are produced. Once they've accumulated, it is believed the 3-hydroxy acylCoA is re-esterified to carnitine, transported back to the cytosol of the cell, and ultimately into circulation. Only patients with LCHADD, and to a lesser extent

TFPD, have long-chain 3-hydroxyacylcarnitines circulating in plasma.[11-13] This makes them a reliable biomarker for incomplete fatty acid oxidation and indicative of LCHADD. This process is depicted in **Figure 1.**

Figure 1

Fat metabolism and hydroxy acylcarnitine production in LCHADD



While elevated long-chain 3-hydroxy acylcarnitines are specifically associated with LCHADD, many of the symptoms of the disorder are similar to other long-chain fatty acid oxidation disorders. Neonates may experience a variety of acute conditions depending on the severity of their phenotype ranging from mild to severe.[1] In the severe phenotype, clinical symptoms usually present within a few days of birth and typically include hypoglycemia, hepatomegaly, encephalopathy, and cardiomyopathy. Intermediate and mild phenotypes may present later in childhood and are characterized by episodes of hypoketotic hypoglycemia, myopathy and/or neuropathy respectively.[1] Early complications can be fatal, if not treated appropriately. As screening, diagnostics, and treatments improve, more patients with LCHADD/TFPD are living into adulthood and chronic complications have emerged. These include recurring rhabdomyolysis; peripheral neuropathy; and chorioretinopathy, though some literature may refer to this as pigmentary retinopathy or even retinitis pigmentosa.

Screening/Diagnosis

Various long-chain mitochondrial β-oxidation defects can be diagnosed using blood spot and/or plasma acylcarnitine profiles. Different acylcarnitines have different fatty acids associated with them, so an elevation in the specific acylcarnitine determines which LC-FAOD is suspected. In the case of LCHADD and TFPD, NBS can quantify the analyte 3-hydroxypalmitoyl carnitine (C16-OH) and 3-hydroxyoleoylcaritine (C18:1-OH). If C16-OH or C18:1-OH values are above the cutoff, the screening laboratory flags the sample as positive, and further biochemical and genetic testing is required to confirm a formal diagnosis of LCHADD/TFPD.[1] It's understood that earlier detection of LCHADD/TFPD can improve outcomes; however, the accuracy of using OH-ACs as a biomarker for LCHADD/TFPD in NBS is varied.[14] In addition to NBS, umbilical cord blood acylcarnitine analysis has shown promising results in even earlier detection of this disorder if there is a known familial history.[3]

Once a blood spot has been flagged as suspicious for LCHADD or TFPD, follow-up testing is conducted to confirm the diagnosis. Subsequent testing includes a plasma acylcarnitine profile or genetic testing for pathogenic variants in *HADHA* or *HADHB*. While testing is in progress, medical nutrition therapy is started to prevent catastrophic events before the test results are completed.

Treatment

Medical nutrition therapy (MNT) for this population strives to reduce β -oxidation of long-chain fats thus reducing the OH-ACs produced. This is achieved through the prevention of prolonged fasting and preventing long-chain fatty acids from being used for energy with small frequent meals. Frequent meals can help avoid hypoglycemic episodes in younger patients.

While there is limited information on specific fasting times specifically for LCHADD, guidelines have been established for VCLADD and MCAD. Fasting times not only vary depending on the age of the patient but also the presence of illness. **Table 1** lists the recommended fasting times by age during well periods.[15-17] When illness occurs and the patient is not eating well, consumption of carbohydrate-rich beverages every 3-4 hours is recommended to maintain blood glucose.[18]

Table 1
Recommendations for fasting times based on age for patients with fatty acid oxidation disorders when well

Age	Maximum during between feedings*
0 to 4 months	3 to 4
4 to 6 months	4 to 6
6 to 9 months	6 to 8
9 to 12 months	8 to 10
Greater than 12 months	10 to 12

*time in hours

Most nutrition recommendations suggest limiting long-chain fatty acid intake because patients with LCHADD/TFPD are unable to oxidize these fats for energy. Current standards suggest limiting long-chain fats to 10% of total energy intake. Patients with LCHADD who consumed 10% of energy or less from LCFA had lower fasting OH-ACs.[19] Low-LCFA diets can be hard to follow and ensure adequate consumption of all other essential nutrients, particularly nutrients associated with higher-fat foods such as essential fatty acids, fat-soluble vitamins, and high-quality protein.

Consuming a low-fat diet puts the individual at risk for essential fatty acid deficiency. Only coming from the diet, essential fatty acids are necessary for many physiological functions including appropriate function of the retina. A 2003 study identifying the optimal diet therapy for patients with LCHADD found that, of the nine participants, μmol/L of α-linolenic acid was deficient in seven participants and μmol/L of linoleic acid was deficient in all nine.[6] The relative weight percentage of the fatty acid species remained normal because of lower amounts of

saturated fatty acids in plasma with a low-fat diet.[6] While not common, clinically significant fatty acid deficiencies have been reported in patients with FAODs. Two cases of polyunsaturated fatty acid deficiency have been reported in VLCADD patients[20] and there have been reports of one LCHADD with persistently low DHA levels despite supplementation of safflower oil.[21] Monitoring plasma fatty acid levels among patients consuming 10% or less of energy from long-chain fat is important to prevent clinically significant deficiencies. EFA deficiency can be especially detrimental in LCHADD patients due to their predisposition to chorioretinopathy. Low plasma DHA is associated with prolonged retinal response time on electroretinogram among children with LCHADD.[6] Because of this, patients are often recommended DHA supplements. Supplementing with DHA increases plasma concentrations and improves the retinal response time on electroretinogram (ERG) suggesting an impact on retinal function.[6] However, beyond this limited data, the impact of DHA supplements on outcomes, including chorioretinopathy is unknown.

In addition to EFA deficiency, this population is also at risk for low fat-soluble vitamin intake. The same 2003 study found that other than vitamin A, dietary intake of fat-soluble vitamins was below the recommendations, but with the addition of a multivitamin, this was resolved.[6] Though intake of water-soluble vitamins and minerals met or exceeded the recommended dietary intake (RDI), it's recommended that LCHADD/TFPD patients also take a multivitamin with vitamins D, E, and K to ensure adequate micronutrient intake.[6]

Because their diet is low-fat, patients make up the difference in calories through an increase in protein or carbohydrate with many patients relying on the latter for caloric intake. A higher protein diet is associated with improved lean body mass (LBM). One study found that a relatively small increase in protein prevented the loss of LBM in participants over the age of 16

yr and promoted LBM gain in participants younger than 16.[4] Improved body composition with increased LBM is associated with maintenance of muscle function and higher energy expenditure.[4]

Recently, a diagnosis of an LC-FAOD has also been associated with decreased resting energy expenditure (REE) and total energy expenditure (TEE) which could potentially lead to an increased prevalence of overweight/obesity in this population.[23] Current basal metabolic rate (BMR) and TEE formulas overestimate energy expenditure among patients with LC-FAODs.[23] This does not appear to be due to differences in body composition, but rather an inherent part of the block in FAO. When Registered dietitians (RDs) estimate energy needs, they could consider a slightly lower total energy requirement among patients with LC-FAODs. In this project, we will take that into account when evaluating reported energy intake compared to estimated BMR.

Though an age-appropriate amount of protein and carbohydrates has been recommended historically, it is now suggested that a slightly increased intake of low-fat, high-quality protein could maintain metabolic control, reduce liver fat without risk of metabolic decompensation, and preserve LBM.[4] During short-term studies[22], patients on a high-protein diet did not experience hypoglycemia, and their insulin sensitivity was normal despite increased adiposity. Whether through a decrease in overall carbohydrate consumption or an increase in protein, a higher protein diet is also associated with a decrease in liver fat.

In addition, patients are often counseled to supplement their diet with medium-chain triglycerides (MCT) since medium-chain fatty acids are oxidized by a different set of enzymes and the medium-chain fatty acid oxidation pathway remains intact. Thus, MCT bypasses the block in long-chain FAO. Current recommendations suggest an additional 10-20% of total energy intake should come from MCT.[6] Patients with LCHADD consuming 20% or more of total

energy from MCT had lower OH-ACs than those consuming less.[18] It is important to note that patients consuming lower LCT and higher MCT had the lowest OH-ACs but independently increasing MCT intake without lowering LCT has not been investigated. Thus, the independent effects of LCT and MCT on blood OH-ACs have not been investigated.

Primarily composed of C8, C10, and C12 fatty acids, MCT oil is the most common supplement to replace long-chain fats in the diet of patients with LC-FAODs. Recently, triheptanoin (Dojolvi®), an odd-chain, seven-carbon triglyceride (C7), was approved by the FDA for the treatment of LC-FAODs. Several studies have proposed that C7 is anaplerotic and enhances the TCA cycle more than medium-chain triglycerides offering a therapeutic advantage over MCT. In a double-blind RCT, triheptanoin improved cardiac function with a lower left ventricular (LV) ejection fraction, smaller LV wall mass, and lower heart rate during moderate-intensity exercise when compared to the traditional MCT (C8) group after 4 months of supplementation. There were no differences in total energy expenditure, body composition, incidence of rhabdomyolysis, or any secondary outcomes between the two treatments.[5] Ketoacids from amino acid catabolism may also replenish the tricarboxylic acid (TCA) cycle thus enhancing energy production similar to the pathway proposed for triheptanon.[22]

Carnitine may be supplemented if a plasma-free carnitine deficiency is present, though this is controversial. Currently, there is little evidence to suggest that there is a benefit to correcting carnitine blood values, but some providers may still recommend supplements. There is no data that carnitine supplements lower or raise plasma OHACs. Further research on the impact of carnitine supplementation in this patient population is needed. In addition to carnitine, patients often take other nutritional supplements. Patients have reported specific nutrient supplements for

eye health among others. In this cohort study, we will describe current supplement regimens for patients with LCHADD/TFPD.

Current dietary recommendations are based on relatively few studies and are primarily derived from case reports and basic disorder knowledge. The association between dietary long-chain and medium-chain fat intake on plasma OH-AC concentrations is primarily based on one observational study that measured the previous 3-day dietary intake with a diet record in 11 participants with LCHADD or TFPD.[6] Additional studies establishing a link between dietary intake of fat and a biochemical marker are needed. Another study found that lower OH-ACs were associated with improved retinal function[7], but this association has not been established with any other chronic complications such as the incidence of rhabdomyolysis.

In addition, the MNT can be difficult to follow and may have negative implications on the quality of life of these individuals. In Williams-Hall, *et al.*[24], researchers conducted a series of interviews with patients with long-chain fatty acid oxidation disorders, caregivers of those patients, and clinicians. They found that in addition to clinical complications, many patients and caregivers reported significant impacts on physical functioning, participation in daily activities, emotional/psychological well-being, and social functioning.[24] Establishing how patients and families are managing current dietary recommendations and the effect of dietary intervention on OH-ACs is important to improve our current recommendations, provide better care, and potentially improve the quality of life for these individuals.

Methods

This study analyzes the dietary data collected for "The Natural History of LCHADD Retinopathy" study, a prospective descriptive study of LCHADD chorioretinopathy. Participants

with a confirmed diagnosis of LCHADD or TFP were eligible to enroll. The study recruitment goal was to include 40 subjects. Exclusion criteria were those who declined to participate. Of the 40 participants enrolled, 35 returned a 3-day diet record. The enrolled participants were 2 to 36 years old. After informed consent/assent was obtained, participants and/or legal guardians were instructed to complete a three-day diet record at home. They returned the diet record in the mail which was then reviewed by the study coordinator and by a registered dietitian (RD). The RD entered the diet information into the ESHA Food Processor for nutrient analysis. For multivitamin supplements, if no brand or amount was reported, a generic multivitamin with minerals was chosen as a default in ESHA. If they reported taking a gummy multivitamin but failed to report the type, a multivitamin without minerals was chosen as the default.

Data Cleaning and Evaluation:

Diet records often underreport total kcals because of forgotten meals, poorly estimated portion sizes, or other omissions on the record. These diet recalls were cross verified with the estimated energy expenditure of that participant to determine the quality/accuracy of the diet record. To do this, the estimated basal metabolic rate (BMR) was calculated using the appropriate Schofield equations listed in **Table 2** for each participant. Then the reported energy intake was divided by BMR and a Goldberg cutoff method[25] used to calculate under- and plausible reported energy intake to determine the reliability of the records. Records with a value greater than 1.1 were considered plausible and those less than 1.1 were considered underreported. Because of the large age range of participants,

Table 2

Schofield Equation for estimating BMR in kcal/day based on weight in kg

Age	**Equation**
Males	
<3	59.512 x W – 30.4
3-9	22.706 x W + 504.3
10-17	17.686 × W + 658.2
18-29	15.057 × W + 692.2
30-60	11.472 × W + 873.1
Females	
<3	58.317 × W - 31.1
3-9	20.315 × W + 485.9
10-17	13.384 × W + 692.6
18-29	14.818 × W + 486.6
30-60	8.126 × W + 845.6

W - weight

macronutrients (kcal, protein, carbohydrates, long-chain fat, and medium-chain fat) will be normalized as kcal and grams of protein per kilogram of body weight and carbohydrate, long-chain fat, and medium-chain fat as percentage of total calories consumed to compare the diets across the population. Micronutrients are expressed as milligrams or micrograms where appropriate.

Data Analysis:

Specific Aim 1: To describe the macro- and micronutrient composition of usual intake in LCHADD/TFPD participants aged 2-36 yr using three-day diet records that include supplements taken.

Hypothesis: Younger participants will consume fewer long-chain fats than older subjects.

The participants were divided into the following age bins: 2-7 years old, 8-15 years old, 16-20 years old, and 21-years and older. Each participant's total calories, kcal/kg, protein, protein g/kg, carbohydrate, fat (long chain and medium chain), and supplement intake were evaluated by descriptive statistics of mean and standard deviation (σ) at a 95% confidence interval (95% CI). The data distribution curve of macronutrients was assessed. All macronutrient distribution was skewed, so we attempted to normalize the data distribution via log transformation after which an ANOVA test was used on the normalized data to evaluate differences between age groups. For macronutrients that remained skewed after transformation, a nonparametric comparison was used. Vitamin intake assessed in this study was analyzed as a percentage of the recommended daily intake (RDI) with and without the addition of supplements. Key variables are summarized in **Table 3**.

Table 3 Key Variables being tested for Specific Aim 1	
Macronutrients	**Micronutrients/Supplements**
Calories	DHA
Protein	Fat Soluble Vitamins
Carbohydrates	Vitamin A
Fat	Vitamin D
Long-chain Triglycerides (LCT)	Vitamin E
Medium-chain Triglycerides (MCT)	Vitamin K
	Water Soluble Vitamins
	Thiamin
	Riboflavin
	Niacin
	Vitamin C
	Carnitine

Specific Aim 2: To determine if a usual diet low in long-chain fatty acids supplemented by MCT oil lowers plasma acylcarnitine levels.

Hypothesis: A diet low in long-chain fatty acids supplemented by MCT oil will have lower plasma acylcarnitine levels.

Fasting blood samples were also collected at enrollment before the 3-day diet record was completed. Plasma was analyzed for acylcarnitines using tandem mass spectrometry.[11] Seven distinct OH-AC species were evaluated by descriptive statistics of mean, standard deviation and a 95% confidence interval. These species are listed in **Table 4.** To create a single variable for the correlations, a principal component analysis (PCA) was used to reduce the data and summarize the variability of the seven specific hydroxy species present within the sample. The PCA value was then used to determine if a relationship between OH-AC and diet exists via Pearson Correlation. Key variables for specific aim 2 are summarized in **Table 4**.

Table 4 Key Variables tested for Specific Aim 2	
Dependent Variables	**Predictor Variables**
PCA1 of: C14:1OH C14:0OH C16:1OH C16:0OH C18:2OH C18:1OH C18:0OH	Long-chain fat intake MCT Intake

Results

Of the 40 participants, 35 returned diet records to be analyzed. There was a relatively even distribution of males and females with 54% male and 46% female. There were 7 participants between 2-7 yr, 12 between 8-15 yr, 9 between 16-21 yr, and 7 above the age of 21. The group between 8-15 years old was the largest. Most (n=21) of the sample was heterozygous for the common c.1528G>C variant and one private variant in *HADHA*, 13 were homozygous for the c.1528G>C variant and one participant had no c.1528G>C allele and was classified as TFPD. **Table 5** summarizes the demographic information of the included participants.

Table 5 Summary of demographic information of participants	
	n (%)
Total	35 (100)
Males	19 (54)
Females	16 (46)
Ages	
2-7 yo	7 (20)
8-15 yo	12 (34)
16-20 yo	9 (26)
21+ yo	7 (20)
Genotype	
No c.1528G>C allele	1 (3)
Homozygous	13 (37)
Heterozygous common c.1528G>C	21 (60)

All participants were taking either MCT oil (n=17) or triheptinoin (n=18). Ninety-one percent of the participants were taking at least one additional supplement with 57% taking

carnitine, 51% taking a multivitamin with minerals, and 46% taking DHA. Detailed supplement information can be found in **Table 6.**

Diet Record Quality: After calculating each participant's BMR using the appropriate Schofield equation for their age and gender, we used the Goldberg method for classifying misreported energy intake.[25] Total reported energy intake (kcal/d) was divided by BMR. We used a conservative cutoff point of 1.1 above the estimated BMR because previous research has demonstrated that patients with LC-FAODs have lower BMR than normal.[23] Records greater than 1.1 were considered plausible, and those less than 1.1 were considered underreported. Of the 35 diet records, only 5 records were considered underreported. An unpaired t-test was performed between the plausible and underreported diet records for both the percent of calories from long-chain fat and the percent of calories from medium-chain fat (MCT oil or triheptanoin) to determine if there was a significant difference between the groups. As shown in **Table 7**, there was a significant difference in LCT and a trend towards a difference in MCT. We considered the possibility of conducting a calorie adjustment for those 5 records but ultimately decided to use the data as reported and no caloric adjustment was made. Only 14% of the records were underreported; 86% were plausible. All diet records were included in the subsequent analysis.

Table 6
Summary of all supplements taken by participants

Medium-Chain Supplement	**n (%)**
MCT Oil	17 (49)
Triheptinoin	18 (51)
Additional Supplements	
Carnitine	20(57)
Multivitamin	18(51)
* B-complex	5(14)
* Riboflavin	2(6)
* Vitamin C	4(11)
* B-carotene	1(3)
* Vitamin D	10(29)
* Vitamin E	6(17)
* Vitamin K	1(3)
DHA	16(46)
Fish Oil	2(6)
Ocuvite	1(3)
None	3(9)

** additional specific nutrient supplements taken in addition to multivitamin*

Table 7
Unpaired t-test between accurate and inaccurate diet records

Fat Type	Plausible Records Mean ± sd	Underreported Records Mean ± sd	p-value	95% CI
LCT	13 ± 5	18.5 ± 5.7	0.0305	0.5545, 10.54
MCT	18.6 ± 9.8	12.3 ± 5.1	0.0882	-15.57, 2.901

CI = confidence interval
LCT = long-chain triglyceride
MCT = medium-chain triglyceride

Specific Aim #1:

Micronutrient intake: We compared the adequacy of micronutrient intake with and without supplements. The mean, standard deviation, and 95% CI of both the diet intake with and without including supplement nutrients are shown in **Table 8.**

Most participants met 100% of the recommended daily intake (RDI) of vitamins assessed without additional vitamin supplementation except for vitamin D, vitamin E, and vitamin K. With supplements, the only vitamin below the RDI was vitamin K. Vitamin A intake, including and excluding supplements, exceeds the RDI by at least 200%. However, the chosen multivitamin default contributed vitamin A as beta-carotene and thus individual intakes were not at risk for meeting the tolerable upper limit (UL). The percent of RDI of each vitamin is found in **Table 9** while **Figure 2** compares the diets including and excluding supplements with the RDI.

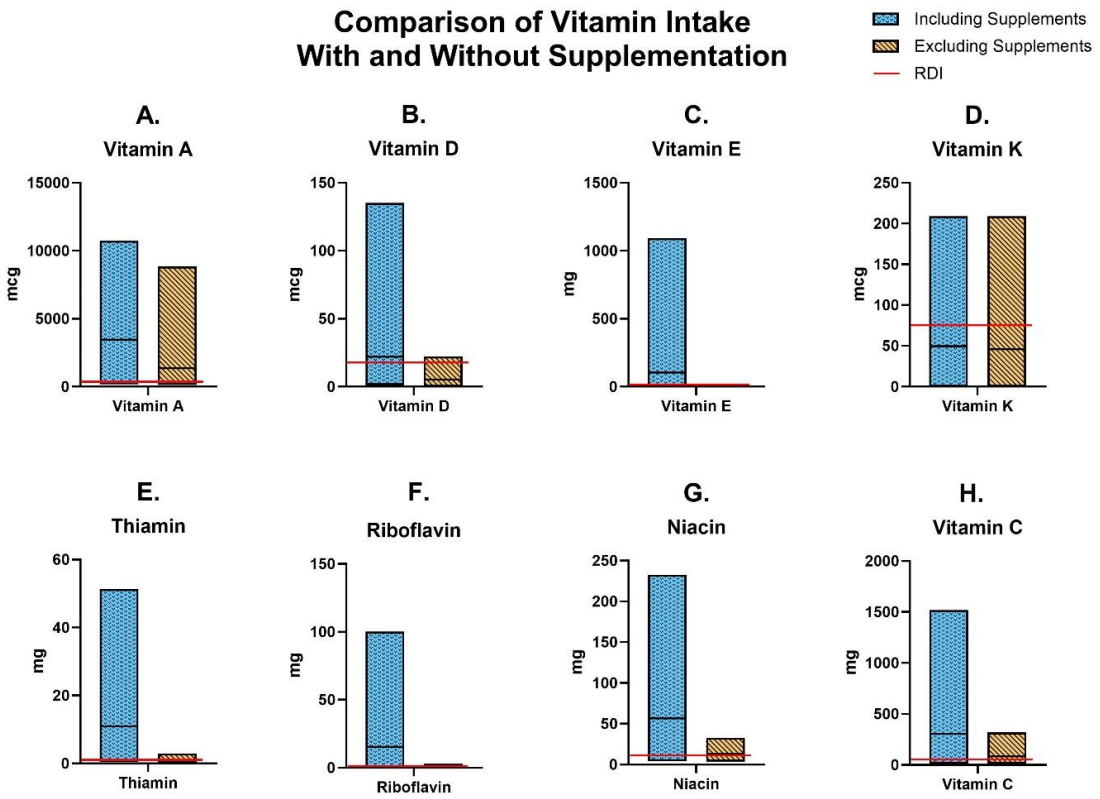
Table 8
Descriptive Statistics of Diet including and Excluding Additional Vitamin Supplements

	Including Additional Supplements		Excluding Additional Supplements	
Nutrient	**Mean ± sd**	**95% CI**	**Mean ± sd**	**95% CI**
Total kcal	1940 ± 572	1744, 2137	1936 ± 572	1739, 2132
Kcal/kg	44 ± 22	36, 51	44 ± 22	36, 51
Total protein (g)	70 ± 30	60, 81	70 ± 30	60, 81
Pro/kg	1.5 ± 0.76	1.2, 1.8	1.5 ± 0.76	1.2, 1.8
Carb (g)	258 ± 101	223, 292	257 ± 101	223, 292
Fat (g)	71 ± 25	62, 80	71 ± 25	62, 80
LCT (g)	29 ± 15	24, 35	29 ± 15	24, 34
MCT (g)	42 ± 25	33, 50	42 ± 25	33, 50
Sat Fat (g)	49 ± 24	41, 58	49 ± 24	41, 57
Poly Fat (g)	4.3 ± 2.9	3.2, 5.3	4.2 ± 2.9	3.2, 5.2
Mono Fat (g)	18 ± 10	14, 21	17 ± 10	14, 21
Vita A (mcg)	3449 ± 3043	2404, 4494	1372 ± 1806	751, 1992
Vita D (mcg)	22 ± 26	13, 31	5.3 ± 4.4	3.7, 6.8
Vita E (mg)	105 ± 202	35, 174	2.5 ± 3.2	1.4, 3.6
Vita K (mcg)	50 ± 64	28, 72	46 ± 61	25, 67
Thiamin (mg)	11 ± 15	5.5, 16	1.1 ± 0.62	0.86, 1.3
Riboflavin (mg)	15 ± 22	7.5, 22	1.2 ± 0.67	0.93, 1.4
Niacin (mg)	56 ± 60	35, 76	13 ± 6.4	10, 15
Vita C (mg)	303 ± 352	182, 423	82 ± 72	58, 107
DHA (mg)	95 ± 150	43, 147		
Carnitine (mg)	389 ± 516	212, 566		

Table 9
Descriptive Statistics of Percent of RDI Met through Diet Including and Excluding Supplements

	Including Supplements		Excluding Supplements	
	Mean ± sd	**95%CI**	**Mean ± sd**	**95%CI**
Vita A	397 ± 330	284, 510	165 ± 202	96, 234
Vita D	111 ± 132	68, 157	28 ± 24	19, 36
Vita E	701 ± 1344	239, 1163	18 ± 24	10, 26
Vita K	43 ± 53	25, 61	40 ± 51	23, 57
Thiamin	892 ± 1204	478, 1306	102 ± 68	78, 125
Riboflavin	1163 ± 1663	592, 1735	101 ± 67	78, 124
Niacin	362 ± 367	236, 488	89 ± 53	71, 108
Vita C	381 ± 406	242, 521	132 ± 176	71, 192

Figure 2

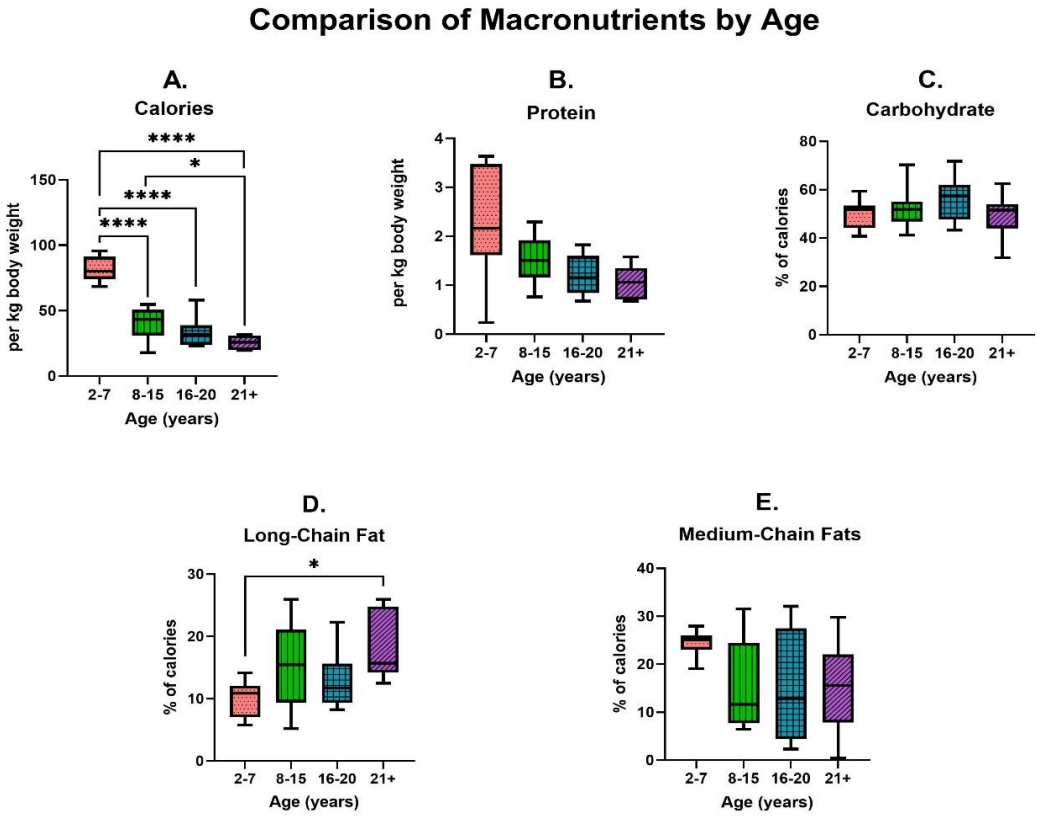


Macronutrient intake: Macronutrient intake was normalized as kcal/kg, protein g/kg of body weight, or as % of total energy from carbohydrates, long-chain, or medium-chain fat. The average % of total energy from LCT of all participants was 14% whereas the average % of total energy from MCT was 18%. The distribution of normalized data was checked, and all variables were skewed. After a log transformation, all variables normalized except for % kcal from MCT.

A one-way ANOVA was used to determine if intake of macronutrients varied across age groups for normalized macronutrient intake values. A Kruskal-Wallis was used for medium-chain fat which remained skewed after transformation. If the overall f-test was significant, a post hoc

analysis was utilized to determine where the significance existed between variables. Kcal/kg was significantly different ($p<0.0001$) as anticipated. Participants in the 2-7 age group consumed more kcal per kg of body weight than all other participants and more long-chain fat as a percent of total kcals than the >21 age group. There was no significant difference in long-chain fat consumption between any other age group. Protein g/kg and the percentage of kcal from carbohydrates, and medium-chain fat were not significantly different ($p=0.3567$, $p=0.803$, $p=0.2495$). **Figure 3** shows the macronutrients by age.

Figure 3



* *p-value less than or equal to 0.05*
**** *p-value less than or equal to 0.0001*

Specific Aim #2:

Fasting blood samples were collected from participants during their evaluation at one of the medical centers. Plasma was shipped to the Mayo Clinic and plasma acylcarnitine profiles were measured by LC-MS/MS as previously described.[26] It should be noted that the 3-day diet record was recorded by the participant or the participant's guardian after they returned home.

Carnitine Supplementation. We compared free carnitine (C0) and acetyl-carnitine (C2) measured in the acylcarnitine profile between participants consuming an oral carnitine supplement and those who were not taking this supplement. An unpaired t-test was performed between the supplemented and non-supplemented participants for both C0 and C2. No significant difference (p=0.7877 [-1.844, 1.410], p=0.3024 [-2.612, 0.8370]) was found in either test suggesting participants consuming oral carnitine supplements did not have higher fasting free or acetyl-carnitine concentrations. The dose of oral carnitine supplements varied. We correlated the dose of carnitine (mg/kg) and free carnitine (C0) concentrations among those taking carnitine supplements. There was no significant correlation (p=0.3754 [-0.8858, 0.5063]) among those taking carnitine and their free carnitine concentration.

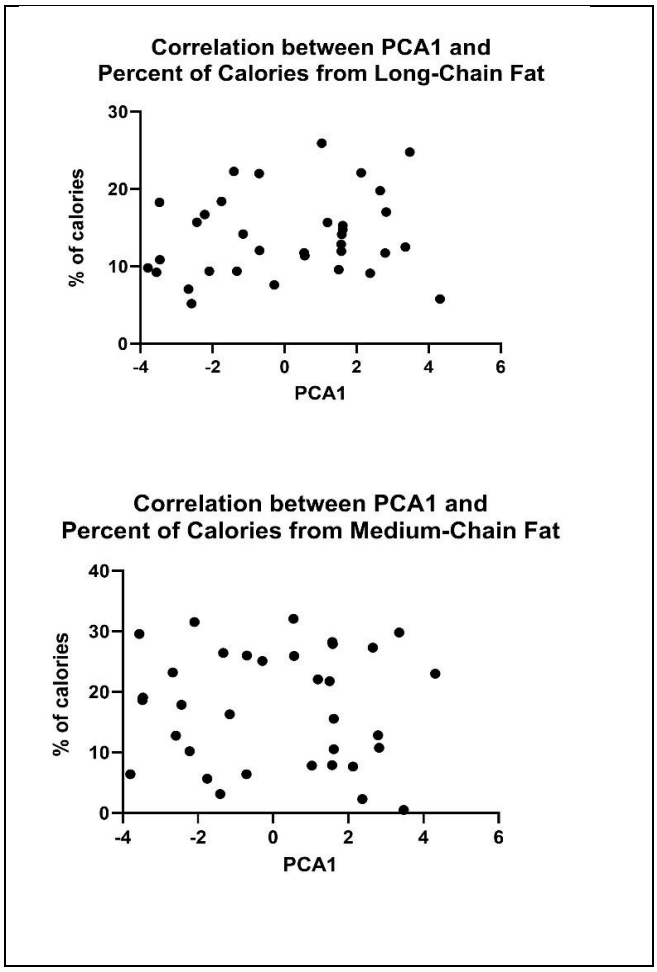
Table 10
Descriptive Statistics of OH-AC Species

OH-AC	**Mean ± sd**	**95%CI**
C14:1OH	0.089 ± 0.8	0.061, 0.14
C14:0OH	0.10 ± 0.9	0.073, 0.l4
C16:1OH	0.13 ± 0.12	0.092, 0.17
C16:0OH	0.28 ± 0.22	0.2, 0.35
C18:2OH	0.12 ± 0.1	0.08, 0.15
C18:1OH	0.45 ± 0.44	0.3, 0.6
C18:0OH	0.3 ± 0.26	0.21, 0.39

OH-AC Concentrations: Summary statistics were used to describe the seven hydroxy-acylcarnitine (OH-AC) species (**Table 10**). A principal component analysis (PCA1) was used to summarize the seven hydroxy species into one data point which was then used to correlate long-

Figure 4

and medium-chain fat intake with the plasma OH-AC concentrations. A Pearsons correlation examined the relationship of PCA1 with the percent of kcal from long- and medium-chain fats and found no significant correlation between either (p=0.2914 [-0.1621, 0.4934], p=0.7881 [-0.3799, 0.2951]). This is shown in **Figure 4.** In this analysis, lower long-chain fat intake or higher MCT intake was not associated with lower OH-AC species contrary to previous reports.[6,19]



Discussion

This study investigated adherence to diet recommendations, micronutrient supplementation, diet's effect on hydroxy-acylcarnitine concentrations, and the effect of carnitine supplementation on free carnitine and acylcarnitine concentrations among participants with LCHADD/TFPD. While some of these findings support previous beliefs, others are contradictory to the current literature. Participants in this study generally followed the established diet recommendation for LCHADD/TFPD. On average, participants followed a low-fat diet with 14% of their energy from LCT compared to about 40% of energy from LCT in the general American Diet. All participants were taking some form of MCT whether that be C7 or C8 at an average of 18% of total energy from either MCT or C7. Only 11 participants of the 35 consumed

less than 10% of their total calories from long-chain fat. The youngest (2-7 yr.) participants consumed less LCT than the oldest (21+ yr) participants but there were no differences among the other groups.

Analysis of the micronutrient intake from food confirmed previous findings that fat soluble vitamin intake in food alone is low. Most participants did not meet the RDI for vitamins D, E, and K from diet alone. 51% of participants are taking a micronutrient supplement, 29% are taking a vitamin D supplement, and 17% are taking a vitamin E supplement. With the supplements, the only vitamin that did not exceed the RDI was vitamin K. Though many vitamins exceeded the RDI, as shown in **Table 9,** none exceeded the tolerable upper limit. These findings suggest that taking a supplement with vitamin D and E can be beneficial to prevent deficiencies of these nutrients.

We found no significant correlation between various diet components and concentrations of long-chain hydroxy-acylcarnitines (OH-AC). This does not mean that diet recommendations for LCHADD/TFD are ineffective. Optimal diet therapy has been associated with retention of retinal function and visual acuity in children with LCHADD or TFPD.[7] Our findings do suggest that OH-ACs are not an effective biomarker for assessing diet adherence. Correlations between intake and lab values have been seen in other smaller studies.[6-7,19] Those studies were conducted in a controlled clinical research environment and included 11 participants. Smaller studies can be susceptible to spurious associations. However, this current study is larger and better reflects the diet habits of patients who are not ill and living in a free environment and may be a better measure of typical status such as when they are seen in an outpatient clinic. Other long-chain fatty acid oxidation disorders have similar observations.[27]

Over half (57%) of the participants in this study were taking some form of carnitine supplement. When comparing the free carnitine (C0) and acylcarnitine (C2) concentrations of the participants taking and not taking carnitine, we found no significant association between carnitine supplementation and either free carnitine or acylcarnitine. There was no significant correlation with carnitine dose and free carnitine concentrations. These findings suggest that supplementation with carnitine does not correspond to a proportional increase in plasma-free carnitine. However, many current practitioners prescribe carnitine supplementation when free carnitine concentrations are deficient. Carnitine supplementation, even low dose supplementation, increases plasma free carnitine into the normal range. It is possible that we do not see a relationship between carnitine supplementation and plasma free carnitine concentrations because once plasma free carnitine is within normal limits, the normal bioregulation of carnitine maintains those concentrations while preventing supraphysiological concentrations from accumulating.

The size and demographic of the population used for this study contribute to the strength of the data presented. The sample used is larger than previous publications[6-7,19] and included participants across a wide age range. Also, diet records and labs were collected in a free-living situation, reflecting a patient's typical status when seen on a routine outpatient basis. The majority of the diet records were considered plausible using the Goldberg method and this study is the first detailed description of the various supplements taken by this population.

Alternatively, the time gap between blood sample and diet record collection is a significant limitation. This study relied heavily on three-day diet records that were collected weeks if not months after blood samples were taken. The time gap between blood draw and 3-

day diet record could potentially hamper our ability to make correlations between dietary intake and blood markers like OH-ACs. This study only assessed the correlation between OH-AC and long- and medium chain fats. Because of this there may be variables that effect these labs (i.e. age and genotype) that were not included in this analysis. In addition, diet records are self-reported diet information. Though we tested for under-reporting, and the majority of the records reported greater than 1.1 estimated basal metabolic rate, there is no way to ensure the records are completely accurate. This is a historical concern about diet data.[28]

In conclusion, this study described the diet of participants with LCHADD/TFPD from a wide range of ages. Participants typically adhered to the current diet recommendations, but adults consume more long-chain fats on average compared to young children. Dietary intake of LCT and MCT were not associated with OH-AC levels in this study, suggesting they may not be appropriate biomarkers to assess diet adherence. However, the diet records were not collected at the same time as the fasting blood samples and there may be other confounding variables that affect OH-AC concentrations. Additional research on the effect of diet on OH-AC concentrations could help better understand and treat individuals with LCHADD/TFPD.

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