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Effects of fasting, feeding and exercise on plasma acylcarnitines among subjects with CPT2D, VLCADD and LCHADD/TFPD

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ABSTRACT

Background: The plasma acylcarnitine profile is frequently used as a biochemical assessment for follow-up in diagnosed patients with fatty acid oxidation disorders (FAODs). Disease specific acylcarnitine species are elevated during metabolic decompensation but there is clinical and biochemical heterogeneity among patients and limited data on the utility of an acylcarnitine profile for routine clinical monitoring.

Methods: We evaluated plasma acylcarnitine profiles from 30 diagnosed patients with long-chain FAODs (carnitine palmitoyltransferase-2 (CPT2), very long-chain acyl-CoA dehydrogenase (VLCAD), and long-chain 3-hydroxy acyl-CoA dehydrogenase or mitochondrial trifunctional protein (LCHAD/TFP) deficiencies) collected after an overnight fast, after feeding a controlled low-fat diet, and before and after moderate exercise. Our purpose was to describe the variability in this biomarker and how various physiologic states effect the acylcarnitine concentrations in circulation.

Results: Disease specific acylcarnitine species were higher after an overnight fast and decreased by approximately 60% two hours after a controlled breakfast meal. Moderate-intensity exercise increased the acylcarnitine species but it varied by diagnosis. When analyzed for a genotype/phenotype correlation, the presence of the common LCHADD mutation (c.1528G > C) was associated with higher levels of 3-hydroxyacylcarnitines than in patients with other mutations.

Conclusions: We found that feeding consistently suppressed and that moderate intensity exercise increased disease specific acylcarnitine species, but the response to exercise was highly variable across subjects and diagnoses. The clinical utility of routine plasma acylcarnitine analysis for outpatient treatment monitoring remains questionable; however, if acylcarnitine profiles are measured in the clinical setting, standardized procedures are required for sample collection to be of value.

1. Introduction

Plasma acylcarnitines are the metabolic product of the import and partial oxidation of fatty acids in the mitochondria. The mitochondrial membrane is impermeable to long chain fatty acids, so the carnitine shuttle is necessary to import fatty acid acyl-CoAs into the mitochondria via carnitine acylcarnitine translocase after conversion to acylcarnitines by the carnitine-palmitoyl CoA transferase 1 (CPT1). Reconversion of acylcarnitines to acyl-CoAs by carnitine-palmitoyl CoA transferase 2 (CPT2) provides very-long chain acyl-CoA dehydrogenase (VLCAD), the initial fatty acid oxidation (FAO) enzyme, and the other β-oxidation enzymes in the mitochondria with the degradable acyl-CoAs to produce cellular energy. Accumulation of specific fatty acyl-CoAs due to a defect in one of the proteins of the carnitine shuttle or the fatty acid oxidation pathway exit the cell as excess acylcarnitines and are measured in an acylcarnitine profile.

The acylcarnitine profile is a powerful screening and diagnostic tool for inherited disorders of fatty acid metabolism characterized by accumulation of specific acylcarnitines. Since the late 1990s, acylcarnitine analysis has become an integral part of newborn screening programs to identify patients early and enable treatment initiation before symptoms occur [1,2]. Subtle elevations in plasma acylcarnitines have

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also been correlated with the presence of insulin resistance [3–5]. Originally described as the Randle or glucose-fatty acid cycle, diabetes researchers have hypothesized that an elevation in acylcarnitines reflect incomplete FAO, mitochondrial overload of fatty acids, and evidence of lipotoxicity leading to insulin resistance [4,6,7].

While it is clear that acylcarnitines are useful for screening and diagnosis, the utility of this biomarker for disease management has not been established. Data on plasma acylcarnitine concentrations in diagnosed and treated patients with FAODs is scarce and often difficult to interpret. It is well known that acylcarnitines are abnormal during acute metabolic decompensation of patients with FAODs along with other biochemical markers like creatine kinase [8–12], but there is large clinical and biochemical heterogeneity across patients. Levels of long-chain (LC) acylcarnitines decrease during initial therapy after diagnosis, which can be helpful in following the efficiency of early treatment. It is more difficult to evaluate the value of long-term monitoring; acylcarnitines can be three-times the upper normal limit with no obvious clinical signs of disease [9,11]. Chronically lower acylcarnitine species are associated with better retinal function among subjects with LCHAD deficiency but not with a decrease in incidence of rhabdomyolysis, or other complications of FAODs [13]. Despite these uncertainties, plasma acylcarnitines are frequently used in follow up of diagnosed patients.

Production of acylcarnitines is related to flux through the FAO pathway and multiple physiological factors regulate this process including lipolysis with fasting, level of fitness with physical activity, and body composition. In patients with FAODs, residual enzyme function might also be an important factor in acylcarnitine production. How these physiologic factors effect FAO flux and plasma acylcarnitine accumulation among patients with FAODs has not been fully explored. Elevated non-esterified free fatty acids (FFA) with accumulation of LC-acylcarnitines after 4 h of fasting in a patient with LCHADD when compared to a healthy sibling suggests normal or increased lipolysis with fasting among patients with LC-FAODs [14]. We have demonstrated fasting acylcarnitines positively correlate with long-chain fat intake and negatively correlated with MCT intake reported in the previous 3-day food diaries among patients with LCHADD suggesting a potential role of diet in plasma acylcarnitine concentrations [15]. Factors associated with elevations of specific acylcarnitines in FAODs remain unclear.

In this report we evaluated acylcarnitine profiles in plasma from previously diagnosed patients with long-chain fatty-acid oxidation disorders (LC-FAODs) collected in a controlled research setting with the purpose of understanding the natural variability in these biomarkers under various physiological conditions such as feeding, fasting and exercise. Additionally, we compared acylcarnitines with plasma FFA as a marker of lipolysis. This was an additional analysis of plasma acylcarnitines from patients diagnosed with LCHAD/TFP deficiency (OMIM# 600890/609015), VLCAD deficiency (OMIM# 201475) and CPT2 deficiency (OMIM# 255110) who participated in a clinical trial of trioctanoin versus triheptanoin [16].

2. Materials and methods

2.1. Study population

Participants diagnosed with LC-FAOD ($n = 32$) were recruited from several family support networks and physician referral. Because of their similar phenotypes, these participants included patients with carnitine palmitoyltransferase-2 deficiency (CPT2D; $n = 11$), very long-chain acyl-CoA dehydrogenase deficiency (VLCADD; $n = 9$), and long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency and trifunctional protein deficiency (LCHADD; $n = 12$) [17]. All participants or their guardians gave written informed consent. Participants younger than 18 years of age also gave assent to participate. This study was approved by the OHSU and UPMC institutional review boards (OHSU eIRB# 817; UPMC eIRB# PRO011020038) and is listed on clinical trials.gov (NCT00654004). Data for this additional analysis was collected during the baseline admission of the study prior to randomization to trioctanoin or triheptanoin; study procedures are outlined in Supplemental Fig. 1A.

Participants were admitted to the OHSU or the University of Pittsburgh's Clinical and Translational Research Center (CTRC) where blood samples were collected for this study. All participants were fed a healthy low long-chain fatty acid (LC-FA) diet that included complex carbohydrates (51–72% of total energy intake), protein (9–17% of total energy intake), low dietary long-chain fat (10% of total energy intake) and medium-chain triglyceride (MCT) oil at 20% of total energy to provide a total of 30% of total energy from dietary fat. Carnitine supplementation was not initiated but maintained among 13 subjects who were already being supplemented (dose 9–82 mg/kg body weight; Supplemental Fig. 2A).

During inpatient admissions, all meals were prepared in the CTRCs Bionutrition Research Kitchen. Participants were asked to eat only the food provided to them by the study and nothing else. Total food intake (weight of food dispensed minus weight of uneaten food returned) was recorded and energy and nutrient intakes were calculated using Pronutra® software by the CTRC bionutrition staff (Viocare Technologies, Inc., Princeton, NJ). Participants were provided approximately 110% of estimated energy needs and told to eat until they were full (ad-libitum feeding). Subjects were fed this controlled diet for 24 h prior to blood sampling and throughout the following 24-h period when blood samples were collected (Supplemental Fig. 1A). Weighed macronutrient intake for 30 subjects is shown in Supplemental Fig. 1B.

An intravenous catheter was placed in a peripheral arm vein to facilitate multiple blood draws. One participant with VLCADD was excluded because of failure to achieve intravenous catheter access. Blood samples were obtained immediately before consuming the meal (after a 10-h fasting) and again at 2 and 4 h post-prandially. Blood samples were also obtained before and after a monitored exercise period consisting of a supervised moderate intensity treadmill exercise test in which subjects walked for 45 min. Blood samples were analyzed for plasma acylcarnitine levels and FFA.

2.2. Plasma acylcarnitine analysis

Blood samples were collected in tubes containing heparin. Plasma was obtained and stored at −80 °C until analysis. All plasma samples were analyzed by flow injection analysis tandem mass spectrometry (FIA-MS/MS) and quantified as previously published [18,19].

2.3. Free fatty acid analysis

Blood samples were collected into EDTA tubes and plasma was separated and a lipase inhibitor added (THL) to prevent ex vivo lipolysis of lipids. Plasma with THL was stored at −80 °C until further analysis. All plasma samples were analyzed by enzymatic colorimetric assay for the quantitative determination of non-esterified fatty acids (NEFA, mEq/L) as previously described (Wako, FLUOstar Omega microplate reader, BMG LabTech).

2.4. Data analysis

Data are reported as mean and standard deviation unless otherwise specified. Change in acylcarnitines over time was analyzed by 1-way repeated measures ANOVA. For all analyses, a $p < 0.05$ was considered statistically significant. The normality of the data was tested using the Anderson-Darling and D'Angostino & Pearson normality tests. For all correlations, we used Spearman correlation analysis for non-normally distributed data. Statistical analysis and graphics were completed using Prism 8.4.2 GraphPad Software Inc. (La Jolla, California).

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Table 1
Subject characteristics, diagnosis and genotype.

Dx	Gender	Age	Age at Dx	Mutations or enzyme assay
CPT2D	F	39	28	common mut not detected*; 20% of control activity
	F	41	40	c.338C > T/c.1238_1239delAG (p.S113L/p.Q413fs)
	F	24	15	c.338C > T/? (p.S113L/?); 16% of control activity
	F	43	42	c.338C > T/c.1239 > 1240delGA (p.S113L/ p.K414fs)
	F	41	31	c.338C > T/c.1511C > T (p.S113L/p.P504L)
	M	9	6	c.338C > T/c.340 + 3A > T (p.S113L/intron 3); 21% of control activity
	F	21	10	common mut not detected*; 7% of control activity
	M	15	13	c.338C > T/c.1239_1240delGA/c.1342 T > C (p.S113L/ p.K414fs/p.F448L)
	M	19	17	c.338C > T/c.1666_1667delTT (p.S113L/p.L556fs)
	F	16	2	c.338C > T/c.1666_1667delTT (p.S113L/p.L556fs)
	F	64	63	c.338C > T/c.338C > T (p.S113L/p.S113L)
VLCADD	M	42	30	c.694G > A/c.1388G > A (p.A232T/p.G463E)
	F	7	NBS	c.1619 T > C/c.1708_1716dupGACGGGGCC (p.L540P/p.D570_A575dup)
	M	38	36	c.343delG/c.1244C > T (p.E115Kfs/p.A415V)
	F	27	15	c.898A > C/c.1097G > A (p.M300V/p.R366H)
	F	39	34	c.637G > A/c.1065_1067delCAT (p.A213T/p.I356del)
	M	42	38	c.637G > A/c.1065_1067delCAT (p.A213T/p.I356del)
	F	23	23	c.1500_1502delCTT/c.1500_1502delCTT (p.L502del/p.L502del)
	M	32	1	c.1322G > A/c.1837C > T (p.G441D/p.R613W)
LCHADD	M	7	3	c.1528G > C/c.703C > T (p.E510Q/p.R235W)
	F	11	NBS	c.1528G > C/c.1528G > C (p.E510Q/p.E510Q)
	M	9	NBS	c.1528G > C/c.1528G > C (p.E510Q/p.E510Q)
	M	11	7 months	c.1528G > C/c.1528G > C (p.E510Q/p.E510Q)
	F	17	8 months	c.1528G > C/c.1528G > C (p.E510Q/p.E510Q)
	M	23	6 months	c.1528G > C/? (p.E510Q/?)
	F	8	NBS	c.1528G > C/? (p.E510Q/?)
	F	24	6 months	c.1528G > C/c.479_482TAG > AATA (p.E510Q/p.I160fs)
	F	16	2	c.901G > A/? (p.G301S/?)
	F	29	NBS	c.1528G > C/c.1528G > C (p.E510Q/p.E510Q)
	M	16	15	c.1150-1G > T/c.208 T > C (intron11/S70P)

Dx = Diagnosis; CPT2D = Carnitine palmitoyltransferase 2 deficiency; VLCADD = Very long-chain acyl-CoA- dehydrogenase deficiency; LCHADD = Long-chain 3-hydroxy acyl-CoA dehydrogenase deficiency; F = Female; M = Male; *common mutations p. 113S > L, p. 50P > H and p. 413 Q > fs were not detected.

3. Results

Subject genotypes are listed in Table 1. Eleven subjects diagnosed with CPT2D were enrolled into the study (8 female and 3 male). Common pathogenic variants were not found in two of the subjects, but enzyme analysis confirmed the diagnosis with 20% or less enzyme activity compared to normal controls. Age ranged from 9 to 64 years with a mean of 30 years of age. Nine patients diagnosed with VLCADD were recruited. One subject was excluded from this analysis due to inability to obtain vascular access. The participating subjects ranged in age from 7 to 42 years of age with a mean of 31 years. Four of the subjects were female and 4 were male, and each had a unique genotype (Table 1). Twelve subjects diagnosed with LCHADD where enrolled in the study. One subject was excluded because consent for secondary research and additional studies outside of the original project was not provided. Nine of these eleven participants were either homozygous or compound heterozygous for the common pathogenic *HADHA* variant c.1528G > C. Two subjects did not carry the common mutation: a female with a c.901G > A/? genotype and a male with a compound heterozygous genotype (c.1150-1G > 1/c.208 T > C). These mutations were previously reported as pathogenic variants associated with a TFP deficiency phenotype [20]. The subjects age ranged from 7 to 29 years of age with a mean of 15 years. Six were female and 5 were male. A total of 30 subjects with a long-chain FAOD are included in this analysis.

The range of selected disease specific acylcarnitine species for the 11 CPT2D subjects is graphed in Fig. 1. After an overnight fast, the highest long-chain acylcarnitine species was C18:1 (mean 0.76 ± 0.35 μmol/L) which was higher than the normal reference range for plasma (0.02–0.38 μmol/L) [19] (Fig. 1A). The other two typical acylcarnitine biomarkers used for CPT2D diagnosis, C16 and C18, were also higher than the reference range (0.03–0.22 μmol/L and 0.01–0.13 μmol/L, respectively). C18:1 decreased by 61% after a breakfast meal and increased more than 223% after exercise (Fig. 1B). However, the individual responses to feeding and exercise in particular, were highly variable (Fig. 1C). Additionally, we compared NBS ratios used to further discriminate cases of FAODs from healthy neonates to the plasma ratios in treated subjects [1]. In the case of CPT2D, the ratio C0/(C16 + C18) was higher than the NBS reference range and the ratio (C16 + C18:1)/C2 was within normal range (Supplemental Table 1).

The acylcarnitine profiles for the 8 VLCADD subjects are graphed in Fig. 2. Fasting plasma long-chain acylcarnitines were within normal limits except for C14:1 (1.39 ± 1.14 μmol/L) which is higher than the reference range of 0.00–0.23 μmol/L (Fig. 2A) [19]. Mean C14:1 acylcarnitine concentration after an overnight 10-h fast fell about 62% after a breakfast meal and increased 133% after moderate intensity exercise (Fig. 2B). Again, the individual responses varied, particularly after exercise (Fig. 2C). Ratios used in NBS to further discriminate VLCADD include C14:1/C2, C14:1/C12:1 and C14:1/C16. Only the C14:1/C16 ratio remained elevated in these treated VLCADD subjects (Supplemental Table 1).

The acylcarnitine profiles of the 11 subjects with LCHADD are graphed in Fig. 3. The most prominent fasting long-chain acylcarnitine was C18:1-OH (0.56 ± 0.52 μmol/L; reference range of 0.00–0.05 μmol/L) (Fig. 3A). C16-OH and C18-OH were higher than the reference range (0.00–0.05 μmol/L and 0.00–0.02 μmol/L, respectively) [19]. C18:1-OH was elevated after an overnight fast, decreased by 59% after breakfast but had minimal change with moderate intensity exercise (Fig. 3B). Unlike the exercise response with CPT2D and VLCADD, this hydroxyacylcarnitine species did not increase much after exercise (20%) and was relatively consistent across subjects (Fig. 3C). When comparing the individual genotype of LCHADD/TFP with acylcarnitine levels, all patients with the presence of the common mutation (c.1528G > C) in at least one allele had consistently higher levels of C16-OH, C18:0-OH (Fig. 3D) and C18:1-OH than the two patients without the common mutation and a TFPD phenotype but the difference was not statistically significant (p = 0.09). Lastly, two

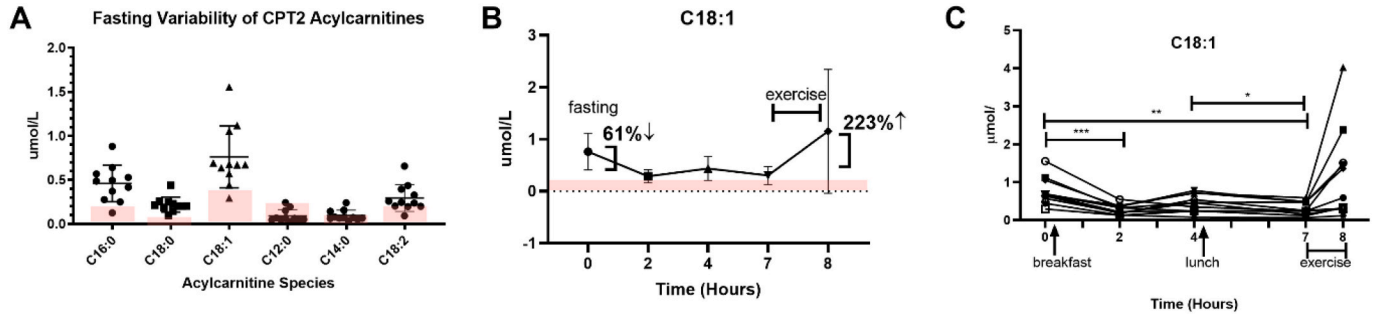

Fig. 1. Fasting and exercise variability of plasma acylcarnitines in patients with CPT2D.
Data are presented as mean ± standard deviation. Reference range is presented as pink shading. A) Selected very long-chain acylcarnitines of CPT2D subjects (n = 11) after 10 h of fasting. C18:1 has the highest concentration overall (0.76 ± 0.35 µmol/L). B) C18:1 is higher than reference range of 0.02–0.38 µmol/L. C18:1 decreased by 61% after a breakfast meal and exercise increased levels by 223%. C) Individual subject values of C18:1 are plotted. C18:1 varies substantially between fasting and 2 h post-prandially and also before and after moderate intensity exercise (1-way repeated measures ANOVA $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acylcarnitine ratios are used to improve diagnostic discrimination of LCHADD in NBS: C16-OH/C16 and C18-OH/C18. These ratios were elevated despite treatment when compared to NBS normal ranges (Supplemental Table 1).

To explore the relationship between lipolysis and LC-acylcarnitines after fasting and exercise, we measured plasma FFA at the same time-points (Fig. 4A). It is well established that lipolysis with fasting and the rise in plasma FFA is directly correlated with a rise in fatty acid oxidation and the production of ketones in the liver [21,22]. However, after exercise, other factors such as uptake of FFA by muscle (FFA disposal) and use of intramyocellular lipid stores impact muscle FAO during exercise in addition to exercise induced lipolysis. FFAs rise with fasting but the response to a controlled fast is variable across subjects. FFA decrease with feeding and begin to rise again with the relatively short fast between meals. The response to exercise is also highly variable. To compare the global pattern of change in FFA to all LC-acylcarnitines across the whole subject population, we summed all the species C14:0 and greater for a sum of LC-acylcarnitines and graphed this total in Fig. 4B. The pattern of change for the sum of the LC-acylcarnitines is similar to the change in FFA, particularly with fasting and feeding. This suggests that lipolysis is a primary factor in the production of acylcarnitine species among patients with LC-FAODs. Next, we correlated the fasting and post-exercise levels of FFA and the sum of the LC-acylcarnitines. FFA and the sum of the LC- acylcarnitines were not normally distributed so we used a Spearman correlation. Fasting FFAs were correlated with the fasting sum of the LC-acylcarnitines (Fig. 4C). However, there was no significant correlation of post-exercise FFAs and the post-exercise sum of LC-acylcarnitines after exercise (Fig. 4D). The data suggest lipolysis measured by plasma FFA is associated with an increase in acylcarnitines after an overnight fast. However, FFA are not correlated with acylcarnitines post-exercise suggesting other factors are more important drivers of partial fat oxidation in response to moderate physical activity.

We also explored the relationships between carnitine supplementation and acylcarnitines. The free carnitine was higher but the fasting sum of LC-acylcarnitines among the 13 subjects with carnitine supplementation was not different from the 17 subjects not consuming carnitine supplements (Supplemental Fig. 2B–C). Creatine kinase (CK) levels were measured after an overnight fast and before and after the moderate intensity treadmill exercise. Fasting CK values in these healthy well controlled subjects were low and there was a lack of a strong correlation with the fasting sum of LC-acylcarnitines. CK levels increased very little (9%) with exercise. There was no correlation between exercise change in CK, change in LC-acylcarnitines or a measure of fitness (exercise speed *grade).

4. Discussion

Acylcarnitine analysis of dried blood spots on filter paper is an effective NBS tool for early detection of FAODs [23]. Confirmation of the diagnosis, follow-up and long-term management use plasma acylcarnitine analysis rather than dried blood spots [24]. As we have shown, plasma acylcarnitine levels vary depending on the physiologic condition. We found that feeding consistently suppresses disease-specific acylcarnitines by approximately 60% and that moderate intensity exercise increases acylcarnitine species but the response to exercise is

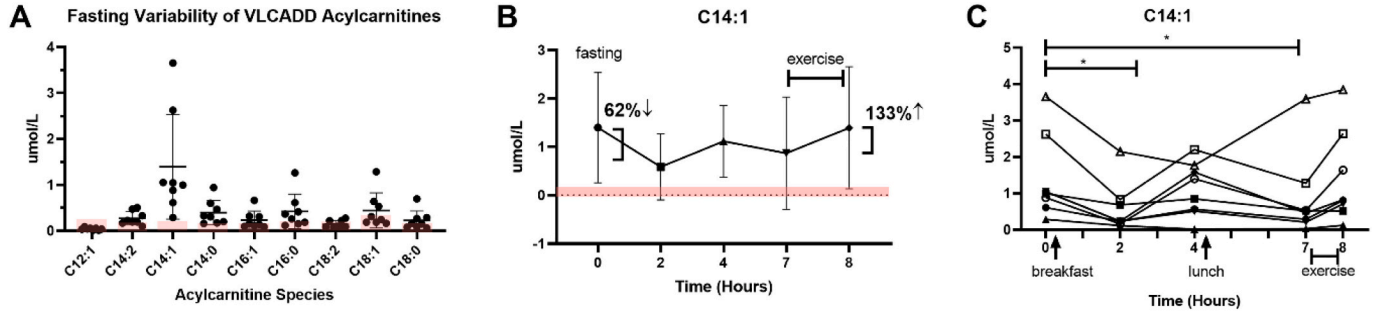

Fig. 2. Fasting and exercise variability of plasma acylcarnitines in patients with VLCADD.
Data are presented as mean ± standard deviation. Reference range is presented as pink shading. A) Selected very long-chain acylcarnitines are shown in VLCADD subjects after 10 h of fasting ($n = 8$). C14:1 was consistently the highest acylcarnitine species: 1.39 ± 1.14 µmol/L (reference range 0.00–0.23 µmol/L). B) Feeding decreased C14:1 level by 62% and exercise increased levels by 133%. In spite of feeding subjects a controlled low fat high MCT diet, mean C14:1 did not normalize. C) Individual responses of C14:1 concentration varied (1-way repeated measures ANOVA, $p = 0.025$). The fasting C14:1 level was significantly higher than the 2-h post-prandial or the pre-exercise sample taken 2 h post lunch. $*p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

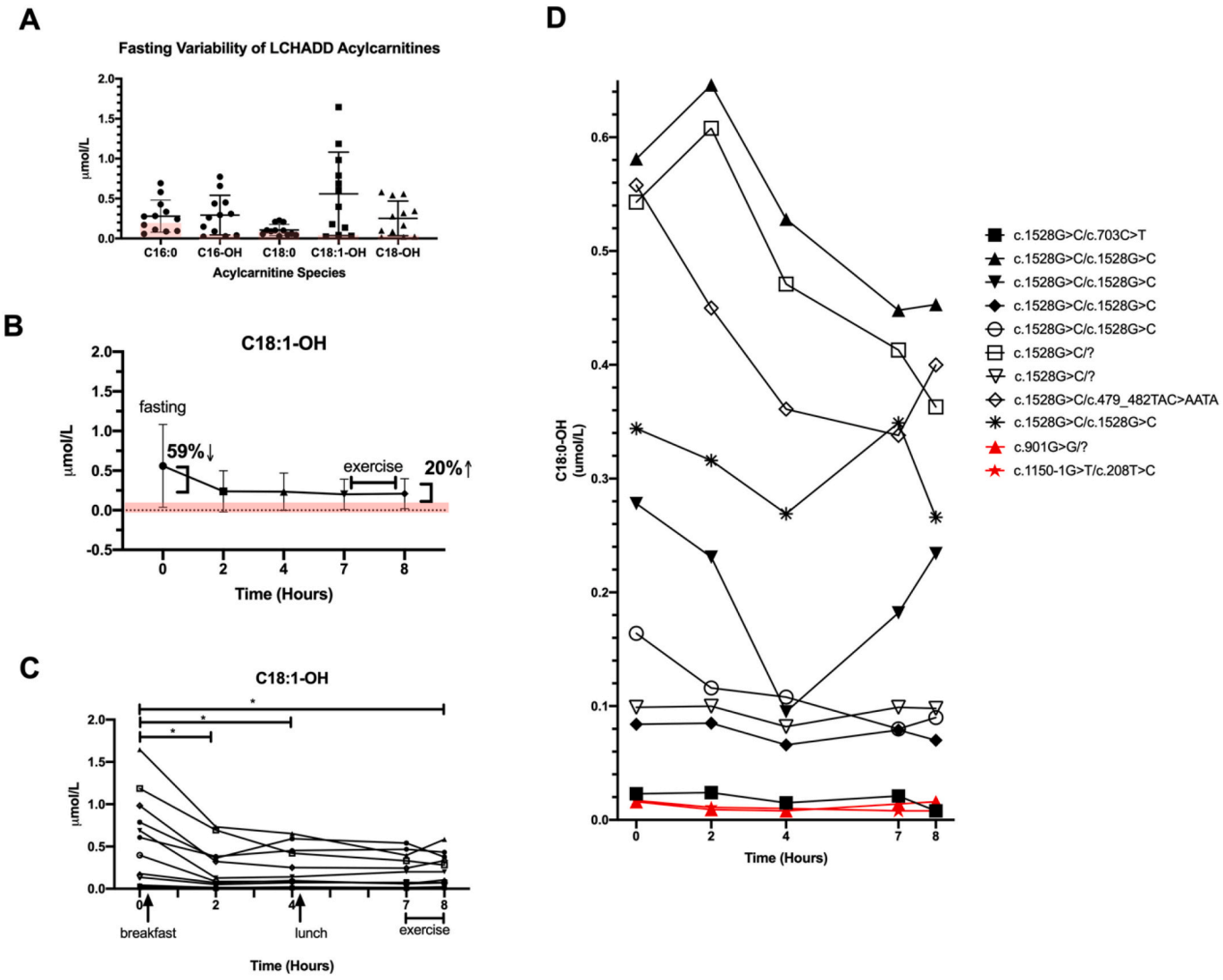

Fig. 3. Fasting and exercise variability of plasma acylcarnitines in patients with LCHADD.
Data are presented as mean ± standard deviation. Reference range is presented as pink shading. A) Selected long-chain acylcarnitines in treated LCHADD subjects (n = 11) after fasting for 10 h. The long-chain 3-hydoxyacylcarnitines remain elevated in spite of treatment; C18:1OH was the highest hydroxylated species. B) Data show C18:1-OH values at fasting, 2 and, 4 h postprandially and before and after moderate exercise (hours 7 and 8) for each of the 11 subjects diagnosed with LCHADD. C18:1-OH ranged from 0.201–0.559 µmol/L. C18:1-OH was elevated after an overnight fast, decreased on average 59% following breakfast but increased by only 20% after moderate intensity exercise. C) Individual subject values are plotted. The fasting C18:1-OH level was significantly higher than the 2-h and 4-h postprandial as well as the post-exercise sample (1-way repeated measures ANOVA *p < 0.05). D) Changes in C18:0-OH are graphed by genotype. Nine out of the 11 subjects had at least one allele with the common mutation (c.1528G > C; LCHADD) with a fasting C18:0-OH mean of 0.229 µmol/L. The 2 subjects without the common mutation (TFPD) are graphed in red and are on the low end on the spectrum with a fasting C18-OH mean of 0.016 µmol/L. The difference among them is not statistically significant (p = 0.09) but may be due to the small sample size and population heterogeneity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

highly variable across subjects and FAOD diagnoses. Disease-specific plasma acylcarnitines are highly variable throughout the day changing with the physiologic states of feeding, fasting and activity so timing of a blood sample collection in relation to physiologic state is critical for the interpretation of the resulting acylcarnitine profile.

Our results suggest that a controlled low-fat diet with MCT and regular meals to avoid fasting lowers plasma acylcarnitines in treated patients compared to levels observed in untreated, undiagnosed patients. Some of the long-chain species were within control ranges and many of the metabolic ratios used for enhanced diagnostic discrimination were within control ranges as well. This was particularly true for subjects with CPT2D where there was an almost complete normalization of LC-acylcarnitines associated with CPT2D in response to treatment (C18:1 remains slightly above normal levels, but the elevation is minimal Fig. 1A). This correlates with the absence of symptoms in these participants. However, despite lower levels than observed in undiagnosed patients, the plasma acylcarnitine profile did not completely normalize in VLCADD or LCHADD subjects. C14:1, an important marker for VLCADD was still elevated and was associated with a high C14:1/C2 ratio (Figs. 2 and Supplemental Table 1). Subjects with LCHADD exhibited elevated long-chain hydroxyacylcarnitines (C16-OH, C18-OH, and C18:1-OH) (Fig. 3). All patients with the common mutation (c.1528G > C) in at least one *HADHA* allele had consistently higher levels of C16-OH, C18-OH and C18:1-OH than the two patients without the common pathogenic variant and a TFP phenotype as previously reported [25] (p = 0.09; Fig. 3D). These findings suggest that mutations that destabilize the protein and result in a loss of mitochondrial TFP expression have low OH-species due to the absence of the hydratase activity. Thus, the plasma acylcarnitine profile will more closely resemble that of a patient with VLCADD [26]. The absence

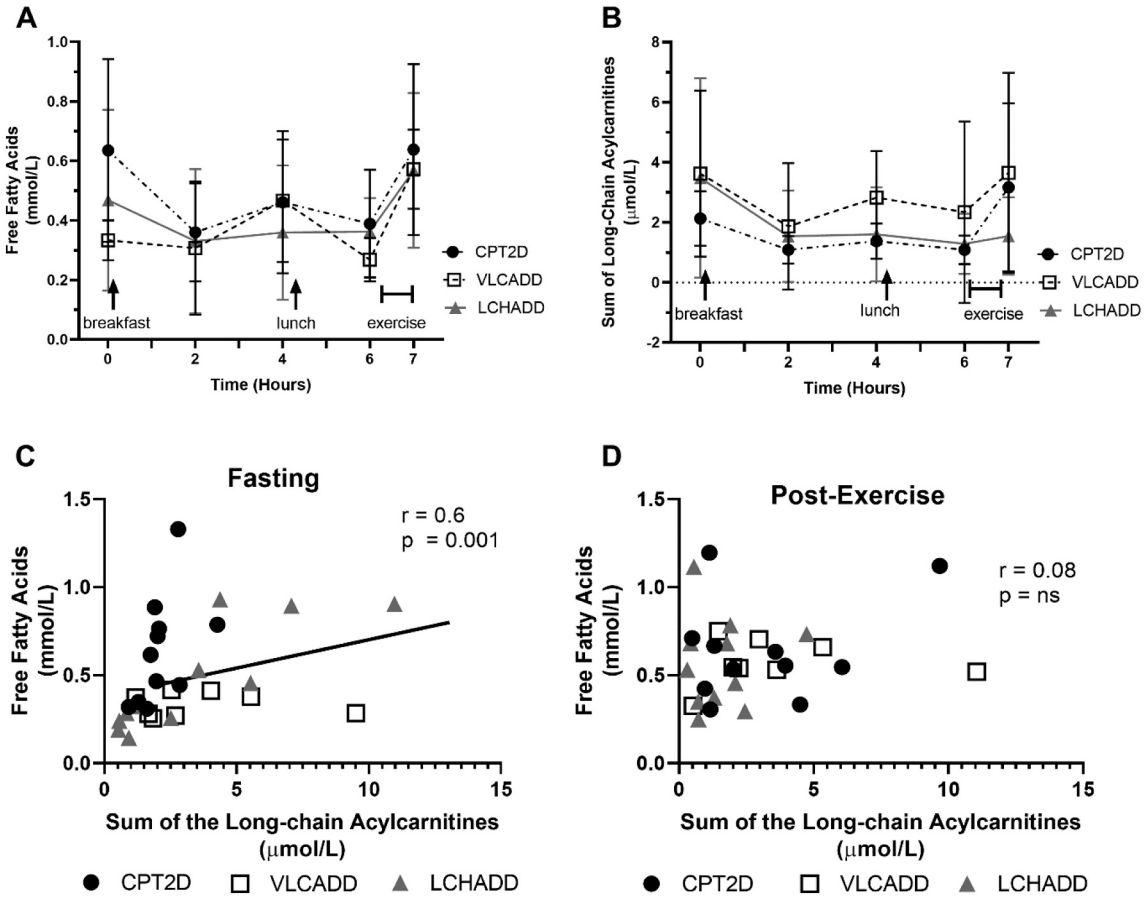

Fig. 4. Fasting and exercise correlations of free fatty acids and the sum of long-chain acylcarnitines by LC-FAODs.
Data are presented as mean ± standard deviation. A) Plasma FFA concentrations in each group measured at the same timepoints as acylcarnitines. FFA rise with fasting and decrease with feeding but the response is variable. The same is observed in response to exercise. B) The sum of LC-acylcarnitines was obtained by adding all the species C14:0 and greater and followed a similar pattern of change with feeding and exercise as seen with FFA. C) There is a positive correlation between the fasting sum of all of the LC-acylcarnitines and free fatty acids. D) However, there is no correlation between the sum of LC-acylcarnitines and free fatty acids after exercise.

of common mutations in our CPT2D and VLCADD cohorts limit our ability to make similar inferences between genotype and acylcarnitines in those disorders, although genotype-phenotype correlations are continuously being reported and this may be possible in the future [27,28].

Carnitine supplementation is common among patients with FAODs. It is hypothesized that increase in plasma availability of carnitine would increase FAO flux in the mitochondria which could potentially be observed by increased plasma acylcarnitine levels. We found that free carnitine was higher but there was no difference in the sum of the LC-acylcarnitines (Supplemental Fig. 2A-C). Another commonly used physiologic marker in FAODs is CK. This study was conducted in healthy, well-controlled subjects and the exercise test was designed to be moderate and not induce rhabdomyolysis. This was corroborated with very low fasting CK levels, the absence of symptomatology with exercise and a small change in CK levels (increase of only 9%). Speed * grade was used as a surrogate for fitness assuming that subjects that walked faster at a greater incline to reach 60% estimated max heart rate would be more fit. There was no strong correlation with LC-acylcarnitines and measures of CK or fitness. All patients tolerated the exercise further supporting the current recommendations for patients with FAODs to be physically active which could improve long-term fitness and exercise tolerance.

Seminal papers have demonstrated that adipose tissue lipolysis, and the rise of FFAs with fasting, drives FAO and ketone production in the liver, increasing the flux through the fatty acid oxidation pathway [29–31]. We were interested in the association of lipolysis and plasma LC-acylcarnitines during fasting. FFAs rise with fasting and decrease with feeding (Fig. 4A). The sum of all LC-acylcarnitines follow a similar pattern in response to fasting and feeding (Fig. 4C). FFAs were positively correlated with the sum of LC-acylcarnitines (Fig. 4B) suggesting that lipolysis is driving flux through the FAO pathway after an overnight fast and increasing plasma LC-acylcarnitines. It further suggests that the source of circulating LC-acylcarnitines after fasting may be the liver, although further study is needed to prove this hypothesis. Feeding suppresses lipolysis and plasma LC-acylcarnitines (Fig. 4A and B), supporting the role of fasting avoidance and frequent meals for lowering LC-acylcarnitines in patients with LC-FAODs.

In contrast, decades of exercise physiology research suggest the activation of the FAO pathway during exercise is more complex. FAO flux is dependent on exercise intensity with moderate intensity exercise activating FAO while high intensity exercise relies on glucose oxidation [32]. Training increases FAO and spares glycogen [33]. Even muscle fiber types effect FAO; slow-twitch fibers are more oxidative and utilize more FA while fast-twitch fibers use almost exclusively glucose. We examined the relationship between lipolysis and moderate exercise but found a very different relationship than we observed with fasting. FFA from lipolysis rose after moderate intensity exercise (Fig. 4A) but the rise in LC-acylcarnitines after moderate exercise was extremely variable, particularly in subjects with CPT2D and VLCADD. Interestingly, the rise was consistently lower among subjects with LCHADD. There was no correlation between FFAs and LC-acylcarnitines after exercise suggesting lipolysis is not the main factor driving FAO with exercise,

and perhaps that the source of the LC-acylcarnitines is not predominately the liver (Fig. 4D). In fact, it has been suggested that adults with VLCADD may have a preponderance of fast-twitch, type 2 muscle fibers that are more glycolytic with moderate exercise than normal healthy controls [34]. Further study is needed to understand the physiologic factors that regulate FAO flux and the appearance of acylcarnitines with exercise among patients with FAODs.

One limitation of this study may also be considered a strength. Subjects were fed a controlled diet and consumed similar macronutrients for the 24 h before and during blood sampling in this study; we are unable to determine the effect of various dietary intakes on plasma acylcarnitines. Although 30 subjects represent a relatively large population of subjects with LC-FAODs, each individual diagnosis had relatively few subjects limiting the statistical power of this predominately descriptive analysis. One of the limitations of the field of FAODs in general is the lack of data correlating outcomes with changes in biomarkers such as acylcarnitines. This may be because there is no relationship and that lowering LC-acylcarnitines does not impact outcomes or decrease disease symptoms. It is also possible that a large prospectively followed cohort is needed to demonstrate such a relationship and so far, this type of natural history study has not been conducted. At this point, the only data demonstrating a relationship between elevations in disease specific acylcarnitines and outcomes is the previously mentioned correlation with elevated hydroxyacylcarnitines and decreased retinal function in subjects with LCHADD [13]. Occasional routine plasma acylcarnitine analysis in LC-FAODs may help establish an individual's “typical” acylcarnitine pattern when healthy which can be valuable in cases of intercurrent illness, or metabolic decompensation.

In summary, we have demonstrated that disease-specific plasma acylcarnitines are highly variable throughout the day changing with the physiologic states of feeding, fasting and activity. Thus, blood collection for acylcarnitine profile analysis in the outpatient clinic setting irrespective of when the patient ate or exercised limits interpretation of the results. The utility of random plasma acylcarnitine analysis for outpatient treatment monitoring may not be beneficial and may not offer useful information beyond the patient's clinical evaluation. However, when acylcarnitine profiles are obtained in the clinical setting, it is recommended that standardized procedures for sample collection (i.e., timing in relation to food intake and physical activity) are strictly followed to improve their clinical interpretation.

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Appendix A. Supplementary data

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