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Elevations of C14:1 and C14:2 Plasma Acylcarnitines in Fasted Children: A Diagnostic Dilemma

Lindsay C. Burrage, M.D., Ph.D.^{1,2}, Marcus J. Miller, Ph.D.¹, Lee-Jun Wong, Ph.D.¹, Adam D. Kennedy, Ph.D.³, V. Reid Sutton, M.D.^{1,2}, Qin Sun, Ph.D.¹, Sarah H. Elsea, Ph.D.¹, and Brett H. Graham, M.D., Ph.D.^{1,2}

¹Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030

²Texas Children's Hospital, Houston, TX 77030

³Metabolon, Inc., Durham, NC 27713

Abstract

Objectives—To test whether follow-up testing for VLCAD deficiency uncovers a diagnosis in patients with elevations of C14:1 and C14:2 plasma acylcarnitines after a controlled fasting study performed for clinically-suspected hypoglycemia and to compare the acylcarnitine profiles from fasted patients without VLCAD deficiency vs. patients with known VLCAD deficiency to determine if metabolite testing distinguishes these groups.

Study design—We performed a retrospective chart review and identified 17 patients with elevated C14:1 and C14:2 plasma acylcarnitine levels after a controlled fast and with testing for VLCAD deficiency (*ACADVL* sequencing or fibroblast fatty acid oxidation studies). The follow-up testing in all patients was inconsistent with a diagnosis of VLCAD deficiency. We compared the plasma acylcarnitine profiles from these fasted patients vs. patients with VLCAD deficiency.

Results—C14:1/C12:1 was significantly lower ($p < 0.001$) in fasted patients vs. patients with VLCAD deficiency. Metabolomics analysis performed in two fasted patients and one patient with VLCAD deficiency demonstrated evidence for upregulated lipolysis and β -oxidation in the fasted state.

Conclusions—Elevations of plasma C14:1 and C14:2 acylcarnitines appear to be a physiologic result of lipolysis that occurs with fasting. Both metabolomics analysis and/or C14:1/C12:1 may distinguish C14:1 elevations from physiologic fasting-induced lipolysis vs. VLCAD deficiency.

Keywords

hypoglycemia; VLCAD deficiency; fasting

Address Correspondence to: Brett H. Graham, M.D., Ph.D., Baylor College of Medicine, Molecular and Human Genetics, One Baylor Plaza, Houston, TX, USA 77030, Phone: 713-798-6209, Fax: 713-798-7773, bgraham@bcm.edu.

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Very long chain acyl-CoA dehydrogenase (VLCAD) deficiency (MIM: 609575) is the most common long chain fatty acid oxidation disorder. The phenotype associated with VLCAD deficiency is variable. The most severe form may present with neonatal cardiomyopathy, hypoglycemia, hepatic dysfunction, multi-organ failure and death. The intermediate form is associated with hypoketotic hypoglycemia and hepatomegaly in early childhood, and the late onset form is characterized by intermittent rhabdomyolysis and exercise intolerance.

An elevation in C14:1 in blood spots is typically the primary analyte for newborn screening (NBS) for VLCAD deficiency¹. However, NBS for this disorder has been fraught with challenges, including normalization of plasma acylcarnitine abnormalities on confirmatory testing which could result in missed diagnoses if further testing is not pursued^{2–6} and elevations of target metabolites in heterozygous or normal individuals^{2, 5}. In addition, patients with normal NBS have occasionally been diagnosed later with VLCAD deficiency^{7, 8}. It has been hypothesized that the best samples for NBS are collected within 36–72 hours of life when infants are still in a catabolic state³. However, by the time confirmatory tests are sent, most infants are in an anabolic state as a result of frequent feeding. Because of these challenges and limitations in confirmation of abnormal NBS by biochemical testing alone, molecular and/or enzymatic testing has been recommended as a second tier testing before deeming an abnormal NBS for VLCAD deficiency a false positive screen^{2, 5, 6, 9, 10}. In addition, prediction tools for interpreting NBS results for VLCAD deficiency using various analytes and analyte ratios rather than a single analyte cut-off have been developed (Region 4 Stork, R4S, post-analytic tools)^{11, 12}.

The genetics service at our center has noted an increase in consultations for C14:1 elevations on plasma acylcarnitine profiles after a controlled fast in children of various ages with history of hypoglycemia. The controlled fast is a diagnostic strategy for investigating hypoglycemia of unknown etiology^{9, 13}. Typically, patients are fasted for a length of time (dependent on age), and blood glucose is monitored. Once hypoglycemia occurs or when the test is terminated, critical specimens are collected. Acylcarnitine profiles, plasma β -hydroxybutyrate, and free fatty acids are included in the critical labs, and the ratio of free fatty acids to β -hydroxybutyrate is used to assess whether a patient has a hypoketotic or hyperketotic response to hypoglycemia⁹. In this study, we present data on 17 patients with hypoglycemia, robust ketone response, and elevated C14:1 and C14:2 levels on plasma acylcarnitine profiles performed at the conclusion of a diagnostic fasting study for hypoglycemia of unknown etiology and with follow-up testing for VLCAD deficiency. Diagnostic testing for VLCAD deficiency was normal in all 17 patients, and these metabolite elevations likely reflected lipolysis after prolonged fasting. To determine whether C14:1 and C14:2 elevations that result from fasting can be distinguished from elevations secondary to VLCAD deficiency, we compare plasma acylcarnitine profiles and metabolomics data from subjects after a fasting study vs. subjects with VLCAD deficiency.

Methods

We identified all patients with elevations in both C14:1 and C14:2 on plasma acylcarnitine profiles that were drawn at Texas Children's Hospital and processed at the Baylor Biochemical Genetics Laboratory between January 2, 2006, and December 15, 2013. For

each of these patients, we performed a retrospective chart review to determine if the plasma sample for acylcarnitine profile was drawn in the setting of a diagnostic fasting study for hypoglycemia, and if so, additional data from the chart were collected including age and sex of the patient, additional laboratories at the time the sample were collected (eg, free fatty acids, β -hydroxybutyrate, glucose), length of fast, results of prior or subsequent plasma acylcarnitine profiles, if performed, and underlying diagnosis, if determined. In addition, if confirmatory testing for VLCAD deficiency was pursued, these results were collected. For metabolomic testing, discarded plasma samples from patients who had testing pursued through the Baylor College of Medicine Biochemical Genetics Laboratory for some other indication were used. All procedures were approved by the Baylor College of Medicine Institutional Review Board (IRB).

Quantitative acylcarnitine analysis was performed as described previously using heparin preserved plasma samples^{14, 15}. Metabolomic profiling was performed using three separate mass spectrometry platforms run in parallel as described previously using heparin or EDTA preserved plasma¹⁶.

Statistical analyses

Data from the metabolomic analysis was converted to a z-score value as described previously¹⁷. The three patient specimens were compared with reference specimens collected from >60 unique individuals who received testing within the biochemical genetics laboratory. Raw mass spectrometry intensity values were scaled to the median value of the entire population, log transformed, and then a z-score was calculated using the average and standard deviation from the control specimens. For the comparison of C14:1/C12:1, a Mann-Whitney test was used.

Results

We identified 123 patients from Texas Children's Hospital with elevated C14:1 and C14:2 acylcarnitines on plasma acylcarnitine profiles. The plasma samples for 34 of these patients were drawn at the conclusion of a diagnostic fasting study for hypoglycemia. Seventeen of these 34 patients had a second test to evaluate for VLCAD deficiency to further assess the plasma acylcarnitine abnormalities identified. For the 17 patients who had further evaluation for VLCAD deficiency, the mean length of the fast was 22 $\pm$ 5 hours with a range of 13.5 to 31 hours, and the mean glucose level at the conclusion of the study was 49 $\pm$ 10 mg/dL. A summary of the study population is presented in Table I.

Because normal ranges for the plasma acylcarnitine levels were adjusted by the diagnostic laboratory during the time period in which samples were collected, we calculated log₂ median scaled values for each acylcarnitine value, and z-scores were calculated using the mean and standard deviation of the entire dataset. Using this ratio, the average z-score for C14:1 for the study group was 3.22 $\pm$ 0.34, and the average z-score for C14:2 was 2.55 $\pm$ 0.67. Analysis of other acylcarnitines also revealed mild elevations in other long chain acylcarnitines including C12, C12:1, C14, and C16:1 (Figures 1–3; Figures 2 and 3 available at www.jpeds.com). Ten of the 17 patients had one or more previous or subsequent plasma

acylcarnitine analyses with normal C14:1 and C14:2 values (Figure 1). The remaining seven patients had only one plasma acylcarnitine profile in our system.

In addition to plasma acylcarnitine profile, other laboratory tests routinely collected at the conclusion of a fasting study include plasma free fatty acids and plasma β -hydroxybutyrate which are used to assess whether the ketone response to the fast is appropriate (Table I). In the 17 patients with elevated C14:1 and C14:2, the ratio of the free fatty acids to β -hydroxybutyrate was 0.7 (range: 0.4 – 1.2; Table I). This ratio suggests a robust ketone response to the fast, and these levels are lower than those observed in a previously published study of a small cohort of patients with fatty acid oxidation disorders who also had fasting studies^{9, 13}. However, this ratio may not be a reliable indicator of a fatty acid oxidation disorder given that high ratios have occasionally been observed in control subjects, and thus, there is overlap in ratios observed in control subjects and those with fatty acid oxidation disorders¹³.

To further evaluate the C14:1 and C14:2 elevations identified at the conclusion of the diagnostic fasting study, molecular testing for VLCAD deficiency was performed in 16 patients. Sequencing of *ACADVL* in 15 of these 16 patients revealed no known deleterious mutations or missense variants of unknown significance in any patient, although one or more benign variants (eg. polymorphisms with frequency greater than 1% and/or deep intronic variants) were detected in 13 patients (data not shown). One of these 15 patients also had immunoblot and fatty acid oxidations studies in fibroblasts sent to an outside commercial laboratory which were normal. One of the 16 patients with molecular testing had whole exome sequencing to evaluate other phenotypes, and no pathogenic variants in *ACADVL* were identified. Furthermore, the exome sequencing did not identify a cause for the hypoglycemia. The one patient with no molecular testing had a normal fatty acid oxidation profile in fibroblasts. Thus, there was no evidence of VLCAD deficiency in any of the 17 patients for whom follow-up testing was pursued, and no patients were even identified with heterozygous pathogenic variants. The underlying diagnosis of most patients in our cohort remains unclear, and four patients have a diagnosis of ketotic hypoglycemia, which is typically a diagnosis of exclusion. Thus, the elevation of C14:1 and C14:2 plasma acylcarnitines in the setting of prolonged fast and hypoglycemia with robust ketone response appears to be physiologic, similar to previous observations^{18, 19}, and not indicative of a diagnosis of VLCAD deficiency.

To further evaluate the plasma acylcarnitine profiles collected after fasting, we compared these profiles with plasma acylcarnitine profiles from patients with a known diagnosis of VLCAD deficiency (n=5 patients with 33 profiles; Figure 1–3). On average, we noted an elevation in C12:1 similar to that observed for C14:1 in the fasted patients. In contrast, although the C12:1 level was variable in subjects with VLCAD deficiency, the magnitude of elevation in C12:1 appeared lower, in general, than the magnitude of the elevation in C14:1. Thus, we hypothesized that C14:1/C12:1 may distinguish fasted patients without VLCAD deficiency from patients with VLCAD deficiency. To test this hypothesis, we compared C14:1/C12:1 in fasted patients vs. patients with VLCAD deficiency and found significantly higher ratios in patients with VLCAD deficiency ($p<0.001$; Figure 4, A). Thus, in this cohort, an elevated C14:1/C12:1 distinguishes an elevated C14:1 secondary to fasting from

elevated C14:1 associated with VLCAD deficiency. Ratios of C14:1 to other plasma acylcarnitines (shorter than C12:1) clearly overlapped between the fasted patients and patients with VLCAD deficiency (Figures 5 and 6; available at www.jpeds.com).

To globally investigate plasma analyte differences between fasting individuals and patients with VLCAD deficiency, we completed a metabolomic profiling analysis as described previously¹⁷. Plasma samples were collected from (i) a patient homozygous for a pathogenic *ACADVL* mutation, c.1182+1G>A (PAT1), (ii) a healthy 32 year old adult male after a 24 hour fast (PAT2), and (iii) a 16 year old female after undergoing a controlled fasting study >24 hours to investigate hypoglycemia of unknown etiology (PAT3) who was also included in the previously described cohort. Analysis of the metabolomics data revealed numerous differences in lipid metabolism between the two groups but similar glucose levels (Figure 4, B). Fasted individuals had high levels of numerous free and dicarboxylic fatty acids, whereas only the monosaturated fatty acid, myristoleate (C14:1), was significantly elevated in the patient with VLCAD deficiency. Long chain acyl-carnitine conjugates were elevated in all patients, but elevations were more striking in the patient with VLCAD deficiency. In contrast, the ketone bodies, 3-hydroxybutyrate and acetoacetate, were much more highly elevated in fasting individuals as compared with the patient with VLCAD deficiency. Lastly, we noted increased C10:0 and C8:0 in the patient with VLCAD deficiency which likely reflects MCT supplementation in the diet as part of the patient's therapy for his underlying diagnosis.

Discussion

Generalized increases in plasma acylcarnitine levels have been observed during fasting studies of both adults and children^{18, 19} and likely reflect increased lipolysis that occurs during fasting. Previous studies have demonstrated that plasma levels of C14:1 acylcarnitine and other long chain plasma acylcarnitines increase in children after a 20 hour fast compared with non-fasted children¹⁸. In our study, the elevations in plasma C14:1 and other long chain acylcarnitine species occurred during a fasting study performed to evaluate known or suspected hypoglycemia. The elevation in C14:1 together with the history of hypoglycemia was concerning for VLCAD deficiency. In 10 of the 17 subjects, repeat plasma acylcarnitine studies were performed outside the setting of a prolonged fast and were normal. However, given reports describing patients with VLCAD deficiency and intermittently normal plasma acylcarnitine profiles³, further diagnostic testing for VLCAD deficiency was performed and was normal in all 17 subjects.

As would be expected in patients without a fatty acid oxidation disorder, we noted that all 17 patients appeared to have a robust β -hydroxybutyrate response after the fast^{9, 13}. Similarly, the metabolomics data provided evidence of a profound ketone response in the two fasted subjects. Although there are few published data describing the level of ketones after a fast in patients with VLCAD deficiency, the ketone response is suspected to be suppressed. However, there is one case report of a child with VLCAD deficiency presenting with large amounts of urine ketones²⁰. Furthermore, in a small study of fasted subjects with a variety of fatty acid oxidation disorders and fasted control subjects, there was overlap in the free fatty acid/ β -hydroxybutyrate ratios observed in control subjects and in subjects with fatty

acid oxidation disorders¹³. Thus, although the ketone response may be helpful in distinguishing fasted patients without VLCAD deficiency from fasted subjects with VLCAD deficiency, the ketone response may not always be reliable for distinguishing elevations in C14:1 that are due to a fast vs. VLCAD deficiency.

As in our studies, prior reports have demonstrated generalized elevations of plasma acylcarnitine species in the setting of a fast¹⁸ which differ from the more specific pattern of elevated long chain acylcarnitine species observed in VLCAD deficiency. The metabolomics data from two fasted subjects revealed generalized elevations of long chain fatty acids, acylcarnitines, and ketones (Figure 4). This more generalized elevation of acylcarnitine species, free fatty acids, and ketones is attributed to lipolysis as fat becomes a primary fuel during fasting once glycogen stores are depleted. Moreover, because of these more generalized elevations in the setting of fasting, C14:1 to C12:1 in plasma acylcarnitine profiles clearly distinguishes the population with elevated C14:1 as the result of fasting vs. patients with VLCAD deficiency in our cohort. This finding is likely due to the fact that there is oxidation of C12 chain lengths in the absence of VLCAD enzyme, and thus, VLCAD enzyme activity is not required for C12 oxidation²¹. We also compared C14:1 with other short chain acylcarnitines (C2 to C12) in the fasted patients vs. patients with VLCAD deficiency (Figures 5 and 6). Interestingly, only C14:1/C12:1 clearly distinguishes the two groups.

One caveat is that our data from patients with VLCAD deficiency are derived from repeated testing from five patients with samples collected at routine clinic visits (non-fasted) and includes the initial diagnostic sample from only two patients. Thus, analysis of a larger population of patients with VLCAD deficiency is necessary to confirm our findings. A second caveat is that we have no data from fasted patients with VLCAD deficiency because prolonged fasting is contraindicated in this disorder, and such testing is not routinely done in this patient population. However, we suspect that this ratio would still distinguish a plasma acylcarnitine profile obtained in a fasted patient with VLCAD deficiency given that the magnitude of elevation of C14:1 should be greater than shorter carbon chains. This hypothesis is supported by a single case report describing a patient with VLCAD deficiency who underwent a fasting study and who had dramatic increases of long chain acylcarnitines limited to chain lengths of C14:1 and higher²².

Our data demonstrate that prolonged fasting complicates the evaluation of plasma acylcarnitine profiles because elevation of C14:1 appears to be a common finding in pediatric patients after a prolonged fast as a result of increased lipolysis and β -oxidation and is not necessarily indicative of a diagnosis of VLCAD deficiency in this setting. When presented with a patient with history of hypoglycemia and elevated C14:1 (acylcarnitine) in plasma after a fast, our data suggest that C14:1/C12:1 may be a useful indicator to distinguish physiologic elevations of C14:1 from fasting vs. VLCAD deficiency. Moreover, our data indicate that global metabolomics data in plasma may be useful for distinguishing patients with a normal physiologic response to fasting vs. patients with VLCAD deficiency given that the generalized elevation of long chain fatty acids, acylcarnitines, and ketone bodies observed in physiologic fasting is distinguishable from the more localized and

dramatic increase in C14:1 and longer chain length acylcarnitine species in VLCAD deficiency.

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Abbreviations

LC-MS	liquid chromatography coupled mass spectrometry
NA	not applicable
NBS	newborn screening
VLCAD	very long chain acyl-CoA dehydrogenase

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Figure 1.

A. Plasma acylcarnitine profiles after fasting show elevation of long chain acylcarnitines. The z-scores for plasma acylcarnitines (C8 and higher) are shown for patients (n=17) with elevated C14:1 and C14:2 after a fasting study. Follow-up studies for VLCAD deficiency were performed and were negative in each of these patients. B. Repeat plasma acylcarnitine profiles in subjects (n=10) are normal in the fed state. One or more repeat plasma acylcarnitine profiles in the non-fasted state were available for 10 of the 17 subjects for whom data were presented in A, and the mean z-scores were normal. C. Plasma acylcarnitine profiles from patients with VLCAD deficiency. The z-scores for the plasma acylcarnitines (C8 and higher) are plotted for five patients with VLCAD deficiency who were identified from our center.

Figure 2.

Z-scores for plasma acylcarnitines (including short chains) for patients (n=17) with elevated C14:1 and C14:2 after a fasting study. The line indicates z-score of 0.

Figure 3.

Z-scores for plasma acylcarnitines (including short chains) for patients with VLCAD deficiency (n=5 patients with repeated measures in some patients). The dark line represents a z-score of 0.

Figure 4.

A. C14:1/C12:1 may distinguish elevations of C14:1 from fasting vs. VLCAD deficiency. C14:1/C12:1 is plotted for subjects with normal VLCAD testing (fasting) and subjects with VLCAD deficiency (VLCAD). The y-axis was log₂ transformed. The dashed line indicates separation between the groups. A Mann-Whitney Test was used for statistical analysis. B. Global lipid metabolism upregulation in fasted individuals but not patients with VLCAD deficiency. The heat map shows z-score values for important lipid biomarkers uncovered by metabolomic profiling. Pat1 is a known patient with VLCAD deficiency, whereas Pat2 and Pat3 are fasted individuals. VLCADD = VLCAD deficiency.

Figure 5.
C14:1 (nmol/L) to C2 (umol/L), C3 (nmol/L), C4 (nmol/L), and C5 (nmol/L).

Figure 6.

C14:1 (nmol/L) to C6 (nmol/L) C8 (nmol/L), C10 (nmol/L), and C12 (nmol/L).

Table 1

Patient fasting data.

	Subjects (Mean $\pm$ SD)	Subjects Range	Normal Range
Sex	5M/12F	NA	NA
Age (months)	62.3 $\pm$ 45.6	14 – 195	NA
Mean Length of fast (hours)	22.1 $\pm$ 5	13.5 – 31.0	NA
Glucose (mg/dl) *	49 $\pm$ 10	31 – 65	70 – 110
Free fatty acids (mmol/L)	2.6 $\pm$ 0.6	1.4 – 3.7	0.5 – 0.9
β-hydroxybutyrate (mmol/L)	3.9 $\pm$ 1.1	1.9 – 5.7	<0.3
Free fatty acids/β-hydroxybutyrate	0.7 $\pm$ 0.3	0.4 – 1.2	NA

* If plasma glucose was not available, whole blood glucose was used

NA = not applicable