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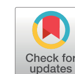
Serum C14:1/C12:1 ratio is a useful marker for differentiating affected patients with very long-chain acyl-CoA dehydrogenase deficiency from heterozygous carriers



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ABSTRACT

Introduction: Various markers, such as C14:1 and the C14:1/C2 ratio, are used as diagnostic markers of very long-chain acyl-CoA dehydrogenase deficiency (VLCADD). However, the levels of these markers in patients with VLCADD overlap with those in heterozygous carriers and even healthy subjects.
Materials and methods: In twenty-three affected patients and 15 heterozygous carriers with VLCADD, the accuracies of C14:1, C14:1/C12:1, C14:1/C2, and C14:1/C16 in dried blood spots (DBS) and serum were statistically estimated.
Results: Among the serum markers, the sensitivity, specificity, positive predictive value, negative predictive value, false-positive rate, false-negative rate, and validity of C14:1/C12:1 were superior to those of C14:1, C14:1/C2, and C14:1/C16, but C14:1/C2 demonstrated a statistical advantage compared with only C14:1 and C14:1/C16. Elevation in serum C14:1/C12:1 was observed in only one heterozygous carrier, whereas almost half of the carriers displayed false positive results for the other markers. Among the DBS markers, although the accuracy of C14:1/C2 was ostensibly the best, no statistical significance was observed.
Discussion: Serum C14:1/C12:1 might be useful for differentiating patients with VLCADD from heterozygous carriers. Although serum C14:1/C2 was significantly useful for the detection of VLCADD, this marker could not distinguish the affected patients from carriers. C14:1/C12:1 might be optimal compared with the other markers.

1. Introduction

Very long-chain acyl-CoA dehydrogenase (VLCAD, EC 1.3.8.9) is located in the inner mitochondrial membrane and is a key enzyme in the first step of mitochondrial long-chain fatty acid β-oxidation [1]. VLCAD deficiency (VLCADD, OMIM 201475) is an autosomal recessive disease [2,3] and is clinically classified into the following three forms: 1) a neonatal-onset (severe) form that exhibits severe cardiomyopathy, respiratory failure, muscle weakness, or hypoketotic hypoglycemia soon after birth, responds poorly to treatment and is often fatal during early infancy; 2) an infantile-onset (intermediate) form that produces hypoketotic hypoglycemia and liver dysfunction, acute encephalopathy, or sudden death triggered by infection, diarrhea, or prolonged fasting during early childhood; and 3) a late-onset (myopathic) form characterized by intermittent attacks of myalgia or rhabdomyolysis after adolescence and normal intelligence [4]. In addition, a “presymptomatic (asymptomatic) form” exists that may remain asymptomatic after detection by newborn screening (NBS) using tandem mass spectrometry (MS/MS) [5].

A study of Japanese NBS using MS/MS found that the incidence of VLCADD is approximately 1:93,000 births [6], which is threefold lower than that in the USA [7]. Although several diagnostic markers of VLCADD in dried blood spots (DBS) have been previously described [8,9], the level of tetradecenoylcarnitine (C14:1-acylcarnitine, the descriptor “acylcarnitine” is subsequently omitted) and the C14:1/C2 ratio (C14:1/C2) are primarily used in NBS. However, these markers sometimes lead to false-positive results because an elevation in C14:1 can often be observed under conditions of hypercatabolism, such as

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Table 1
Number of patients and samples.

	Total no. of Pt	No. of patients (samples)	
		serum	DBS
Patients with VLCADD			
Severe form	3	3 (3)	3 (6)
Intermediate form	8	8 (35)	7 (54)
Myopathic form	7	7 (30)	5 (13)
Presymptomatic form	5	4 (20)	4 (21)
Total	23	22 (88)	19 (94)
Heterozygous carriers			
Detected by NBS	8	7 (44)	8 (47)
Parents or siblings of patients	7	3 (3)	6(6)
Total	15	10 (47)	14 (53)
Control		185 (185)	251 (251)

No: number, Pt: patients, VLCADD: very long-chain acyl-CoA dehydrogenase, DBS; dried blood spot, NBS: newborn screening.

poor sucking [10], and has even been identified in carriers of VLCADD [11,12]. Although it has been previously reported that the C14:1/C12:1 and C14:1/C16 ratios are useful for the diagnosis of VLCADD [13–15], studies evaluating the serum (s-)C14:1/C12:1 ratio are limited. Here, we report that the s-C14:1/C12:1 ratio is a precise marker, particularly for discriminating VLCADD patients from heterozygous carriers and healthy subjects.

2. Materials and methods

2.1. Subjects and acylcarnitine analysis

2.1.1. Subjects

As shown in Table 1, the subjects in this study included 23 patients with VLCADD (22 cases with 88 serum samples and 19 cases with 94 dB samples) and 15 heterozygous carriers (14 cases with 51 dB samples and 10 cases with 47 serum samples), who were detected at Shimane University between 1994 and 2015, and a control group (251 dB samples and 185 serum samples) recruited in September and October 2014. All patients and heterozygous carriers were genetically and/or enzymatically diagnosed, except for a heterozygous 9 (H9) who was diagnosed without an examination because of patient 14 (P14)'s mother. The clinical courses of some patients (P6, P9, P10, P12, and P18) have been previously reported [16–19]. In this study, the clinical forms were determined based on the clinical symptoms and course but not the genotype; the "severe form" was diagnosed regardless of symptoms if death occurred during early infancy; the "intermediate form" was defined based on hypoglycemia attacks during infancy or childhood; and the "myopathic form" was diagnosed based only on myopathy regardless of the onset age. The controls were screened for inherited metabolic diseases at Shimane University but were found to be normal.

The samples from the VLCADD patients were collected at different time points, such as during the neonatal period, during the acute phase and under stable conditions, or after glucose infusion and supplementation with L-carnitine, but did not include NBS samples. This study was approved by the Institutional Review Board of Shimane University (#20190517-1).

2.1.2. Blood acylcarnitine analysis

Acylcarnitine (AC) in DBS or serum was analyzed using MS/MS (API-3000; Applied Biosystems, Foster City, CA, USA) after butyl-derivatization of the samples as previously described [20,21]. Briefly, methanol (200 μL) containing an isotopically labeled internal standard (Kit NSK-A/B, Cambridge Isotope Laboratories, Cambridge, UK) was added to one disc of a DBS (3.1 mm in diameter) or 10 μL of serum, and the mixture was incubated for 30 min; 150 μL of the supernatant after centrifugation were butylated with 50 μL of 3 N n-butanol–HCl. The sample was dissolved in 100 μL of 80% acetonitrile, and the concentrations of ACs were quantified by MS/MS.

2.1.3. Diagnostic markers

The values of C14:1, C14:1/C12:1, C14:1/C2, and C14:1/C16 in DBS and serum were investigated. The cutoff values, except for the cutoff value of C14:1/C16, were determined based on the standard values used at Shimane University (s-C14:1, < 0.2 μM; s-C14:1/C12:1, < 4.0; s-C14:1/C2, < 0.02; b-C14:1, < 0.3 μM; b-C14:1/C12:1; < 4.0, and b-C14:1/C2, < 0.013). Because C14:1/C16 was not used as a diagnostic marker for VLCADD at our institution, the cutoff values (s-C14:1/C16, < 2.0 and b-C14:1/C16, < 0.1) used in this study were determined based on a previous report [15].

2.2. Data analysis

In cases with several samples obtained at different times, the bottom values were extracted as the representing values in the patients with VLCADD, and the top values were extracted in the heterozygous carriers and controls to evaluate the accuracy of the diagnostic markers. Our data were statistically analyzed using a univariate analysis by JMP pro 12 (SAS Institute Inc., North Carolina, USA). The false-positive rate, false-negative rate, and validity were measured as one minus specificity, one minus sensitivity, and real positive plus real negative divided by the number of samples, respectively.

3. Results

Table 2 shows the range of each AC marker, the ratios, and free carnitine (C0) in serum and DBS. All markers failed to perfectly make a diagnosis of VLCADD even in the severe and intermediate forms.

3.1. AC analysis of serum

In the patients with VLCADD, the median (and range) s-C14:1, s-C14:1/C12:1, s-C14:1/C2, and s-C14:1/C16 were 1.29 μM (0.14–10.79, cutoff < 0.2), 9.56 (1.78–52.24, cutoff < 4.0), 0.113 (0.014–0.881, cutoff < 0.02), and 2.20 (1.24–8.21, cutoff < 2.0), respectively (Table 3). In the heterozygous carriers, the median s-C14:1, s-C14:1/C12:1, s-C14:1/C2, and s-C14:1/C16 were 0.32 μM (0.12–0.69), 3.08 (1.00–5.98), 0.017 (0.008–0.029), and 1.59 (0.67–3.77), respectively, while in the controls, the median s-C14:1, s-C14:1/C12:1, s-C14:1/C2, and s-C14:1/C16 were 0.08 μM (0.00–1.39), 1.33 (0.00–6.32), 0.006 (0.000–0.031), and 0.65 (0.00–4.13), respectively.

Regarding the accuracy of these markers, s-C14:1 failed to detect three (P5, P9, and P14) of the 22 patients with VLCADD and overdiagnosed 33 cases (6 of the 10 carriers and 27 of the 185 controls) as shown in Table 4. The sensitivity and specificity of s-C14:1 were 0.864 and 0.831, respectively (Table 5). s-C14:1/C12:1 failed to detect two patients (P21 and P23) and overdiagnosed 8 cases (one carrier and seven controls). The carrier (H5) who showed a false positive was examined eight times, but his s-C14:1/C12:1 exceeded the cutoff value only once. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), false-positive rate, false-negative rate, and validity of s-C14:1/C12:1 were the best compared with those of s-C14:1, s-C14:1/C2, and s-C14:1/C16 in our samples using the cutoffs at our institution. s-C14:1/C2 missed two patients (P5 and P21) and overdiagnosed nine cases (4 carriers and 5 controls). s-C14:1/C2 also displayed the highest sensitivity and lowest false-negative rate (0.909 and 0.091, respectively). Regarding s-C14:1/C16, six patients (P2, P5, P13, P14, P18, and P21) were false negative, while 14 cases (4 carriers and 10 controls) were false positive.

The ROC (receiver operating characteristic) curve of s-C14:1/C2 showed the largest AUC (area under the ROC curve) (Fig. 1A). The AUC of s-C14:1/C2 was significantly larger than that of s-C14:1 and s-C14:1/

Table 2
Ranges of serum and DBS markers in VLCAD deficiency patients, heterozygous carriers, and controls.

		Serum (μmol/L)						DBS (μmol/L)					
ID	genotype	s-C0	s-C14:1	s-C14:1/C12:1	s-C14:1/C2	s-C14:1/C16	No. of sample	b-C0	b-C14:1	b-C14:1/C12:1	b-C14:1/C2	b-C14:1/C16	No. of sample
		(20–60)*	(< 0.2)*	(< 4.0)*	(< 0.02)*	(< 2.0)*		(20-60)*	(< 0.3)*	(< 4.0)*	(< 0.013)*	(< 0.1)*	
Severe form													
P1[a]	G152S/A333Cfs*26	28.00	3.15	44.37	0.43	6.06	[1]	19.44–28.78	1.15–7.52	15.91–43.87	0.09–0.69	0.44–1.26	[2]
P2[a]	G152S/A333Cfs*26	33.87	5.12	52.24	0.88	1.92	[1]	2.74–33.53	0.70–4.63	20.00–38.58	0.18–0.93	0.40–0.85	[3]
P3	IVS9 + 1G > C/G185S	144.43	10.79	20.75	0.39	2.86	[1]	69.40	4.50	15.52	0.13	1.39	[1]
Intermediate form													
P4	IVS7-18TAG del/L172V	23.85–27.80	1.61–5.8	9.67–14.64	0.06–0.18	5.13–6.70	[2]	15.98–31.83	0.55–2.91	6.27–17.31	0.06–0.42	0.66–2.80	[4]
P5	A333Cfs*26/A416T	42.11-61.29	0.14–3.33	6.05–13.84	0.011–0.21	1.64–9.21	[5]	15.36–35.69	0.10–6.97	3.11–25.25	0.003–0.88	0.07–6.51	[22]
P6	F113*/K382Q	34.18-95.85	1.39-14.00	25.09–97.50	0.10–0.91	2.01–7.95	[16]	18.86–45.93	0.69–5.03	12.32–48.59	0.10–0.57	0.63–3.18	[15]
P7	P89S/A536Yfs*15	85.42-163.45	2.02-5.83	11.22–48.50	0.13–0.30	2.10–5.21	[5]	NA	NA	NA	NA	NA	(0)
P8	K382Q/G447R	30.96–51.06	1.29–7.06	8.94–19.55	0.28–0.28	3.08–6.45	[2]	18.38	0.74	13.45	0.22	1.57	[1]
P9[b]	L243F/V547M	44.76	0.17	5.48	0.02	2.30	[1]	21.24–27.85	0.12–4.62	1.67–9.24	0.02–0.21	0.16–1.43	[3]
P10[b]	L243F/V547M	13.85–86.79	0.34–7.23	9.44–22.59	0.02–1.17	2.42–11.30	[3]	7.45–54.49	0.15–7.81	4.16–28.46	0.02–0.70	0.16–1.99	[8]
P11[#1]	G222R/IVS9 + 3G > T	52.29	7.69	29.58	0.43	6.25	[1]	39.44	11.50	34.72	1.01	8.27	[1]
Myopathic form													
P12	R229*/K382Q	21.48-60.28	3.10-12.33	36.05–100.59	0.20–0.94	2.80–7.10	[6]	15.2–28.77	0.96–5.05	22.32–42.82	0.09–0.68	0.93–2.54	[6]
P13	A333fs*26/R450H	8.21-130.71	1.91–15.99	25.13–78.80	0.24–2.20	1.59–5.74	[12]	5.26–7.91	0.86–1.45	22.10–25.40	0.13–0.23	1.06–1.75	[2]
P14[#2]	G354Hfs*4/A416T	40.14-63.07	0.14–3.88	7.00–11.09	0.02–0.27	1.89–5.62	[2]	18.48	0.39	3.97	0.05	0.35	[1]
P15	L172P/A180T	5.93–39.29	1.28–2.38	26.44–63.79	0.20–0.43	4.10–4.87	[3]	7.92–13.52	1.20–1.73	10.56–25.37	0.24–0.29	1.38–1.84	[2]
P16	K264E/K264E	46.80	2.54	9.40	0.20	7.06	[1]	NA	NA	NA	NA	NA	(0)
P17	K264E/M437V	84.96–146.20	1.26–4.37	13.69–20.66	0.06–0.22	2.06–7.67	[3]	51.76–60.7	1.02–1.63	12.83–21.73	0.05–0.11	0.43–0.76	[2]
P18[#3]	E285G/V400M	46.43–84.51	0.47–0.51	8.25–12.14	0.05–0.06	1.24–2.04	[3]	NA	NA	NA	NA	NA	(0)
Presymptomatic form													
P19	NA	25.88	1.15	7.67	0.14	8.21	[1]	NA	NA	NA	NA	NA	(0)
P20	K264E/n.d.	35.71–37.63	0.25–0.91	5.68–6.41	0.03–0.11	2.08–7.00	[2]	23.71–28.60	0.50–1.80	6.44–8.57	0.05–0.07	1.08–1.45	[2]
P21	C607S/C607S	45.91–47.65	0.25–0.32	1.78–5.55	0.018–0.03	1.39–3.72	[3]	35.20–41.28	0.10–0.13	0.97–2.66	0.003–0.006	0.07–0.13	[3]
P22	F112L/R450H	NA	NA	NA	NA	NA	(0)	23.08	0.30	5.56	0.016	0.65	[1]
P23	T260P/C607S	30.25–53.97	0.40–3.78	2.67–10.21	0.02–0.37	2.00–10.27	[14]	18.17–30.66	0.27–1.12	1.65–8.52	0.015–0.10	0.36–1.15	[15]
Heterozygous carrier													
H1	C237R	25.06–65.39	0.05–0.28	0.42–3.15	0.005–0.018	0.24–1.55	[9]	22.72–36.96	0.06–0.18	0.59–8.29	0.003–0.010	0.07–0.22	[9]
H2	G439fs*462	30.29-52.44	0.04-0.38	0.84–2.77	0.005–0.026	0.43–3.18	[11]	17.55–39.32	0.03–0.15	0.60–3.36	0.004–0.019	0.05–0.29	[10]
H3	G289R	32.08–55.73	0.10–0.39	1.09–3.00	0.005–0.015	0.42–1.64	[5]	24.09–43.93	0.12–0.16	0.58–1.71	0.003–0.006	0.07–0.14	[5]
H4	IVS9 + 1G > C	51.52–54.41	0.10–0.14	2.38–3.25	0.006–0.008	0.64–0.83	[2]	24.47–39.32	0.04–0.17	0.57–2.66	0.002–0.008	0.04–0.15	[3]
H5	D466Y	33.20–52.66	0.11–0.64	1.47–5.98	0.008–0.029	0.30–3.77	[8]	20.59–51.91	0.07–0.38	0.93–5.89	0.003–0.020	0.04–0.29	[8]
H6	W249L	52.21–65.76	0.11–0.35	1.08–3.54	0.007–0.026	1.10–2.92	[5]	19.29–36.86	0.05–0.09	0.61–1.31	0.003–0.006	0.06–0.10	[5]
H7	IVS9 + 1G > C	NA	NA	NA	NA	NA	(0)	12.18	0.23	1.07	0.009	0.08	[1]
H8	K299del	35.73–58.08	0.12–0.69	0.90–3.45	0.012–0.020	1.46–2.68	[4]	29.36–47.76	0.10–0.43	0.28–3.91	0.001–0.009	0.02–0.22	[5]
H9[#2]	NA	61.30	0.16	1.60	0.016	1.45	[1]	NA	NA	NA	NA	NA	(0)
H10	A333Cfs*26	NA	NA	NA	NA	NA	(0)	48.26	0.02	0.89	0.005	0.03	[1]
H11	R385W	NA	NA	NA	NA	NA	(0)	40.77	0.03	n.d.	0.009	0.07	[1]
H12[#1]	IVS9 + 3G > T	NA	NA	NA	NA	NA	(0)	50.39	0.18	1.16	0.008	0.19	[1]
H13[#1]	G222R	NA	NA	NA	NA	NA	(0)	53.62	0.26	1.98	0.009	0.17	[1]
H14[#3]	V400M	102.37	0.12	1.00	0.009	0.67	[1]	56.74	0.08	1.00	0.003	0.06	[1]
H15[#3]	E285G	116.91	0.11	1.45	0.014	1.14	[1]	53.31	0.10	1.43	0.006	0.13	[1]
Control		12.24–112.40	0.00–1.39	0.00–6.32	0.000–0.031	0.00–4.13	(185)	8.23–38.30	0.02–0.25	0.24–5.5	0.001–0.010	0.01–0.10	(251)

No., number; DBS, dried blood spots; VLCAD, very long-chain acyl-CoA dehydrogenase; a, b, sibling; #1, #2, #3, parent.
Underlined text indicates values lower than the cutoff in VLCAD deficiency patients or higher than the cutoff in heterozygous carriers and controls.
NA, not available; n.d., not detected.
* , reference values are used at Shimane University.

Table 3
Results for the markers in DBS and serum.

	Serum							DBS						
	VLCADD					Hetero-zygous carrier (n = 10)	Control (n = 185)	VLCADD					Hetero zygous carrier (n = 14)	Control (n = 251)
	Severe (n = 3)	Inter-mediate (n = 8)	Myopathic (n = 7)	Pre symptomatic (n = 4)	Overall (n = 22)			Severe (n = 3)	Inter-mediate (n = 7)	Myopathic (n = 5)	Pre symptomatic (n = 4)	Overall (n = 19)		
s-C14:1 (< 0.2 μM)								**b-C14:1 (< 0.3 μM)**						
Median	5.12	1.34	1.28	0.32	1.29	0.32	0.08	1.15	0.55	0.96	0.29	0.69	0.18	0.07
Range	3.15–10.79	0.14–7.69	0.14–3.1	0.25–1.15	0.14–10.79	0.12–0.69	0.00–1.39	0.70–4.50	0.08–11.5	0.39–1.20	0.1–0.5	0.08–11.50	0.02–0.43	0.00–0.25
Mean	6.35	1.83	1.53	0.51	2.11	0.33	0.12	2.12	1.98	0.89	0.29	1.36	0.18	0.08
SD	3.24	2.31	0.99	0.37	2.60	0.19	0.15	1.70	3.90	0.27	0.14	2.57	0.12	0.04
s-C14:1/C12:1 (< 4.0)								**b-C14:1/C12:1 (< 4.0)**						
Median	44.37	9.56	13.70	4.18	9.56	3.08	1.33	15.91	6.27	12.83	3.60	10.56	1.71	1.13
Range	20.75–52.24	5.48–29.58	7.00–36.05	1.79–7.67	1.78–52.24	1.00–5.98	0.00–6.32	15.51–20.00	1.67–34.72	3.97–22.33	0.97–6.44	0.97–34.72	0.89–8.29	0.00–5.50
Mean	39.12	13.06	18.00	4.45	16.62	2.92	1.57	17.14	10.81	14.36	3.65	11.24	2.67	1.25
SD	13.38	8.27	10.39	2.35	13.63	1.33	1.01	2.03	10.61	7.04	2.38	8.75	2.14	0.71
s-C14:1/C2 (< 0.02)								**b-C14:1/C2 (< 0.013)**						
Median	0.426	0.08	0.203	0.029	0.113	0.017	0.006	0.128	0.060	0.089	0.016	0.060	0.008	0.004
Range	0.392–0.881	0.011–0.429	0.024–0.239	0.018–0.148	0.014–0.881	0.008–0.029	0.000–0.031	0.090–0.180	0.003–1.006	0.049–0.244	0.003–0.057	0.003–1.00	0.003–0.020	0.000–0.010
Mean	0.567	0.128	0.142	0.056	0.179	0.018	0.007	0.133	0.205	0.114	0.023	0.131	0.009	0.004
SD	0.223	0.136	0.085	0.053	0.202	0.007	0.005	0.037	0.335	0.072	0.020	0.218	0.005	0.002
s-C14:1/C16 (< 2.0)								**b-C14:1/C16 (< 0.1)**						
Median	2.86	2.36	2.07	2.04	2.20	1.59	0.65	0.44	0.627	0.93	0.50	0.63	0.14	0.03
Range	1.92–6.06	1.65–6.25	1.24–7.06	1.39–8.21	1.24–8.21	0.67–3.77	0.00–4.13	0.40–1.39	0.07–8.27	0.35–1.38	0.07–1.09	0.07–8.27	0.03–0.29	0.00–0.10
Mean	3.61	3.12	2.96	3.42	3.19	1.98	0.82	0.74	1.65	0.83	0.54	1.06	0.15	0.03
SD	1.77	1.56	1.88	2.78	1.98	1.01	0.59	0.46	2.75	0.39	0.38	1.76	0.08	0.01

DBS; dried blood spot, VLCADD; very long-chain acyl CoA dehydrogenase deficiency, SD; standard deviation.
These data were extracted from the bottom values of VLCADD patients and the top values of heterozygous carriers and controls in the patients who were examined several times.

Table 4
Primary data for each marker in serum and DBS.

s-C14:1 (< 0.2 μM)	Patients	Not affected			b-C14:1 (< 0.3 μM)	Patients	Not affected		
		Carriers	Control				Carriers	Control	
Positive	19	6	27	52	positive	14	2	0	16
Negative	3	4	158	165	negative	5	12	251	268
	22	10	185	217		19	14	251	284

s-C14:1/C12:1 (< 4.0)	Patients	Not affected			b-C14:1/C12:1 (< 4.0)	Patients	Not affected		
		Carriers	Control				Carriers	Control	
Positive	20	1	7	28	positive	14	2	5	21
Negative	2	9	178	189	negative	5	12	246	263
	22	10	185	217		19	14	251	284

s-C14:1/C2 (< 0.02)	Patients	Not affected			b-C14:1/C2 (< 0.013)	Patients	Not affected		
		Carriers	Control				Carriers	Control	
Positive	20	4	5	29	Positive	17	2	0	19
Negative	2	6	180	188	Negative	2	12	251	265
	22	10	185	217		19	14	251	284

s-C14:1/C16 (< 2.0)	Patients	Not affected			b-C14:1/C16 (< 0.1)	Patients	Not affected		
		Carriers	Control				Carriers	Control	
Positive	16	4	10	30	Positive	17	10	1	28
Negative	6	6	175	187	Negative	2	4	250	256
	22	10	185	217		19	14	251	284

C16 ($p = 0.013$ and $p = 0.008$, respectively). The other markers revealed no significant differences. While reanalyzing the accuracy of these markers using Youden Index (ideal cutoff values in our samples), the sensitivity, NPV, and false-negative rate of s-C14:1/C2 were the best, while the specificity, PPV, false-positive rate, and validity of the C14:1/C12:1 ratio were the best (data not shown).

3.2. AC analysis of DBS

In the DBS samples obtained from the patients, the median (and range) b-C14:1, b-C14:1/C12:1, b-C14:1/C2, and b-C14:1/C16 were 0.69 μM (0.08–11.50, cutoff < 0.3), 10.56 (0.97–34.72, cutoff < 4.0), 0.060 (0.003–1.00, cutoff < 0.013), and 0.63 (0.07–8.27, cutoff < 0.1), respectively (Table 3). In the heterozygous carriers, the median b-C14:1, b-C14:1/C12:1, b-C14:1/C2, and b-C14:1/C16 were 0.18 μM (0.02–0.43), 1.71 (0.89–8.29), 0.008 (0.003–0.020), and 0.14 (0.03–0.29), respectively. In the controls, the median b-C14:1, b-C14:1/C12:1, b-C14:1/C2, and b-C14:1/C16 were 0.07 μM (0.00–0.25), 1.13 (0.00–5.50), 0.004 (0.000–0.010), and 0.03 (0.00–0.10), respectively.

The sensitivity and false-positive rate of almost all DBS markers were lower than those of the serum markers, while the specificity, PPV, and false-negative rate of the DBS markers tended to be higher (Table 5). In particular, b-C14:1 and b-C14:1/C12:1 showed a high false-negative rate. Although the accuracy, including the sensitivity and specificity, of b-C14:1/C2 was superficially the best, the AUC of b-C14:1/C2 was the lowest (Fig. 1B). Although the AUC of b-C14:1/C16 was the largest, no DBS markers revealed statistical significance.

4. Discussion

Our study indicated that s-C14:1/C12:1 and s-C14:1/C2 are useful markers for the detection of VLCADD. In particular, s-C14:1/C12:1 contributed by excluding the false positive of heterozygous carriers. The false-positive rate in the heterozygous carriers was 10% by s-

Table 5
Accuracy of markers in serum and DBS.

	Serum				DBS			
	s-C14:1	s-C14:1/C12:1	s-C14:1/C2	s-C14:1/C16	b-C14:1	b-C14:1/C12:1	b-C14:1/C2	b-C14:1/C16
Cutoff	(< 0.2 μM)	(< 4.0)	(< 0.02)	(< 2.0)	(< 0.3 μM)	(< 4.0)	(< 0.013)	(< 0.1)
Sensitivity	0.864	**0.909**	**0.909**	0.727	0.737	0.737	**0.895**	**0.895**
Specificity	0.831	**0.959**	0.954	0.928	**0.992**	0.974	**0.992**	0.958
PPV	0.365	**0.714**	0.690	0.533	0.875	0.667	**0.895**	0.607
NPV	0.982	**0.989**	0.989	0.968	0.981	0.981	**0.992**	0.992
False-positive rate	0.169	**0.041**	0.046	0.072	**0.008**	0.026	**0.008**	0.042
False-negative rate	0.136	**0.091**	**0.091**	0.273	0.263	0.263	**0.105**	**0.105**
Validity	0.834	**0.954**	0.949	0.908	0.975	0.958	**0.986**	0.954

Cutoffs are used at Shimane University.
The most effective values are shown **in bold**.
PPV, positive predictive value; NPV, negative predictive value.
Validity is calculated as (real positive + real negative)/(number of sample).

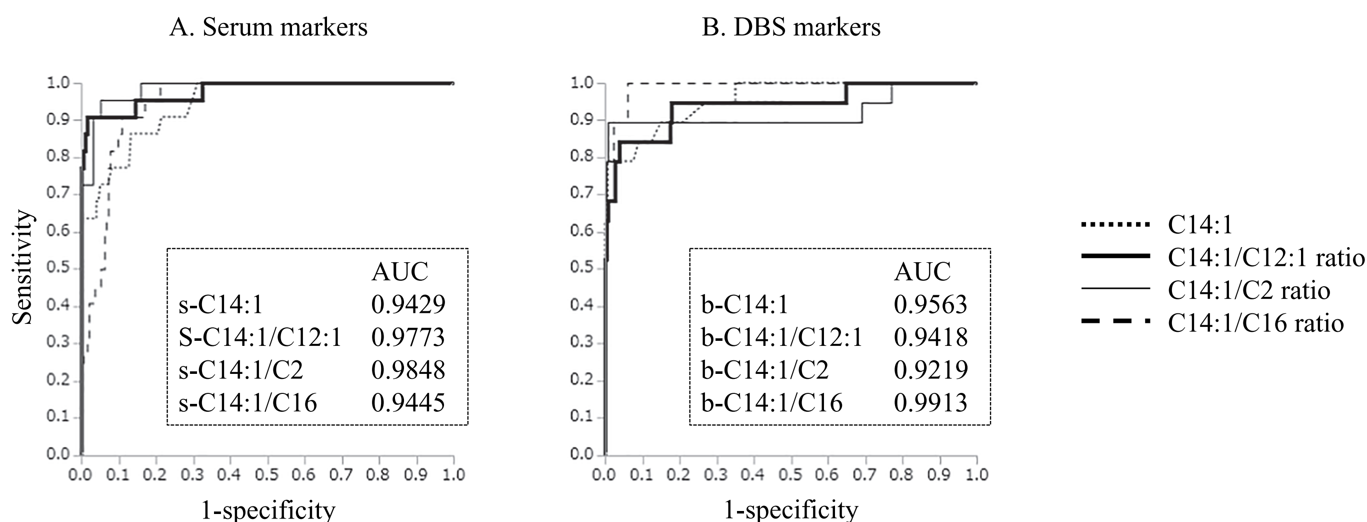

Fig. 1. ROC curve of serum and dried blood spots markers.
ROC, receiver operating characteristic; AUC, area under the ROC curve; DBS, dried blood spots; s-, serum; b-, blood (DBS).

C14:1/C12:1, whereas those detected by other serum markers were 40–60%. Although only s-C14:1/C2 demonstrated a statistical advantage, s-C14:1/C2 displayed a high false-positive rate in the heterozygous carriers. Moreover, the combination of s-C14:1 and s-C14:1/C12:1 may further decrease the false-positive rate because the s-C14:1 levels did not increase in five of the seven controls whose s-C14:1/C12:1 was elevated in our study. In contrast, the combination of s-C14:1 and s-C14:1/C2 may not be effective because the s-C14:1 levels were also increased in all nine cases (4 carriers and 5 controls) whose s-C14:1/C2 was elevated.

Our results further suggest that the serum and DBS AC profiles are not always related to clinical severity, although the determination of clinical forms was slightly arbitrary in this study; we defined the clinical forms based on phenotypes but not genotypes. While some markers of myopathic and presymptomatic patients sometimes were higher than those of severe and intermediate patients, the levels of all markers were sometimes within the normal range under a stable condition in the patients with not only presymptomatic and myopathic forms but also the intermediate form (Tables 2 and 3). For instance, s-C14:1/C12:1 misdiagnosed only two presymptomatic patients (P21 and P23), and s-C14:1/C2, which was a statistically significant marker, missed one intermediate and one presymptomatic patient (P5 and P21). Additionally, the levels of s-C14:1/C16 were not increased even in a severe VLCADD patient (P2). Although it has been previously reported that affected patients are occasionally missed in NBS due to the deficient sensitivity of DBS [22–24], physicians should be aware that serum markers can also miss the severe form of VLCADD if a single marker is used. To prevent false-negative findings, overviewing the AC profiles, using several markers and/or their combinations, and avoiding the collection of samples soon after feeding/eating may be useful.

More useful DBS markers were not found in our study. As previously reported [21,25,26], our results also indicated that the DBS markers displayed less sensitivity than the serum AC markers. Although b-C14:1/C2 seemed to be valuable in our study, in practice, false negatives caused by poor sucking are occasionally detected using this marker in NBS [10]. In comparing our data with the data of Region 4 stork (R4S) projects [9], our results were very different from the results of R4S (data not shown), maybe because the sampling time and conditions were different; the R4S data were based only on NBS, but our samples were collected at acute phase and/or under stable conditions during outpatient follow-up and did not include NBS. Therefore, our results could not be directly applied in NBS. Nevertheless, b-C14:1/C2 and b-C14:1/C16 might be relatively useful for preventing false-negative results compared with b-C14:1 and b-C14:1/C12:1.

C14:1/C12:1 is considered a biochemically precise marker for the detection of VLCADD. This is because C14:1 is accumulated and C12:1 is not accumulated in VLCADD. In detail, although the metabolism of C14:1 derived from oleic (C18:1-) acid was partially compensated by medium-chain acyl-CoA dehydrogenase, this compensation is not sufficient in VLCADD [27]. Conversely, C12:1, the next metabolite of C14:1, can be metabolized by both enoyl-CoA isomerase and enoyl-CoA hydratase, even in VLCADD [28].

Finally, although we presented the accuracy of AC markers in this study, the accuracy, including the sensitivity, specificity, PPV, NPV, false-positive rate, false-negative rate, and validity, could vary depending on the cutoff values. Furthermore, we collected samples at different times and under different conditions, and the frequency of the AC analysis also differed in each patient. Although C14:1/C12:1 should be theoretically useful regardless of DBS or serum, we could not demonstrate usefulness of b-C14:1/C12:1 in this study. Further studies are necessary to determine whether the C14:1/C12:1 ratio in serum is definitely useful in real clinical practice.

5. Conclusion

The serum C14:1/C12:1 ratio was an excellent biochemical diagnostic marker of VLCADD and particularly useful in differentiating patients with VLCADD from heterozygous carriers.

Contributions of individual authors

K. Yamada designed the study, wrote the initial draft of the manuscript, and acquired the funding. Y. Osawa, H. Kobayashi, and Y. Hasegawa participated in the analysis and interpretation of the data and revised the manuscript. S. Fukuda, S. Yamaguchi, and T. Taketani critically revised this draft for important intellectual content and provided final approval to submit this article.

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Ethical approval

This study was approved by the Institutional Review Board of Shimane University (#20190517-1).

Informed consent

All procedures were performed in accordance with the ethical standards of the responsible Committee on Human Experimentation (institutional and national) and the Helsinki Declaration of 1975 as revised in 2000. Informed consent was not obtained from all cases in this study, but we confirmed the intention of our subjects with opt-out.

Declaration of Competing Interest

None.

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