Original Article

Increased acylcarnitine ratio indices in newborn screening for carnitine-acylcarnitine translocase deficiency shows increased sensitivity and reduced false-positivity

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Background: Carnitine-acylcarnitine translocase (CACT) deficiency is a rare autosomal recessive metabolic disorder of mitochondrial long-chain fatty acid oxidation. Newborn screening via tandem mass spectrometry (MS/MS) technology enables early diagnosis. However, previous analyses of MS/MS data of patients showed that some results were misdiagnosed because they did not show typical acylcarnitine profiles of CACT deficiency. This study aimed to identify additional indices to assist the diagnosis of CACT deficiency.

Methods: To evaluate the acylcarnitine profile and the acylcarnitine ratios of individuals with CACT deficiency, the MS/MS data of 15 patients diagnosed via genetic testing were retrospectively analysed. The sensitivity and false-positive rates of primary acylcarnitine markers and ratio indices were validated using the data from 28,261 newborns and 53 false-positive cases. Additionally, the MS/MS data of 20 newborns carrying the c.199-10T>G mutation in *SLC25A20* and 40 normal controls were compared to verify whether the carriers had abnormal acylcarnitine concentrations.

Results: The acylcarnitine profiles from 15 patients were classified into three categories using C12, C14, C16, C18, C16:1, C18:1, and C18:2 as the primary diagnostic markers. The first category represented a typical profile (P1–P6). The second category for patients P7 and P8 showed a significant decrease in the C0 level and a normal concentration of long-chain acylcarnitines. The third category for patients P9–P15 showed the presence of interfering acylcarnitines. The second and third categories may have been misdiagnosed. An acylcarnitine ratio analysis showed that C14/C3, C16/C2, C16/C3, C18/C3, C16:1/C3, and C16:1-OH/C3 were significantly increased in all 15 patients. The verification of 28,261 newborn screening results showed that the false-positive rate of ratios, except for (C16 + C18)/C0, was lower than that of acylcarnitine indices (0.02–0.08% *vs.* 0.16–0.88%). None of the single long-chain acylcarnitines could separate patients from the false-positive cases; however, all ratios produced good discrimination between the two groups.

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Conclusions: Based on the primary acylcarnitine markers alone, CACT deficiency can be misdiagnosed in newborn screening. The ratios of the primary markers (C16 + C18:1)/C2, C16/C2, C16:1/C3, and C16:1-OH/C3 can facilitate the diagnosis of CACT deficiency, thereby increasing sensitivity and reducing false-positivity.

Keywords: Carnitine-acylcarnitine translocase deficiency; newborn screening; ratio; tandem mass spectrometry (MS/MS); acylcarnitine

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Introduction

Carnitine-acylcarnitine translocase (CACT) is a key enzyme in mitochondrial fatty acid oxidation; it mainly catalyses the exchange of acylcarnitine and free carnitine on both sides of the mitochondrial inner membrane and is essential for the transport of long-chain acylcarnitine into the mitochondrial matrix for beta-oxidation (1,2). CACT deficiency (OMIM#212138) is a rare autosomal recessive metabolic disorder that occurs due to mutations in the CACT gene (*SLC25A20*) (OMIM#613698) (3). The classic phenotype of CACT deficiency is characterised by hypoketotic hypoglycaemia, neurological abnormalities, arrhythmias, liver dysfunction or hepatomegaly, and myopathy; most patients become symptomatic in the neonatal period with a rapidly progressive deterioration and a high mortality rate (2,4,5). Metabolic characteristics include low free carnitine (C0) levels and abnormal acylcarnitine profiles with marked elevation in the levels of long-chain acylcarnitines (6,7). Newborn screening via tandem mass spectrometry (MS/MS) is an important approach for the early diagnosis or indication of CACT deficiency. However, since the acylcarnitine profile characteristics in CACT deficiency are the same as those of carnitine palmitoyltransferase II deficiency (6), definitive identification requires the measurement of CACT enzyme activity or the mutation analysis of *SLC25A20* gene.

The incidence of CACT deficiency is relatively low in the Chinese population; the predicted incidence is 1 in 76,894 in Hunan (8), 1 in 100,000 in Guangzhou (9), and 1 in 60,000 in Hong Kong (10). Therefore, CACT deficiency is not commonly encountered during newborn screening. The primary acylcarnitine markers selected in the diagnosis of CACT deficiency are C12, C14, C16, C18, C16:1, C18:1, and C18:2 (6,7). A long-term diagnosis of inborn errors of metabolism found that not all patients with CACT deficiency showed matching acylcarnitine profiles. Due to the presence of interfering acylcarnitine that primary acylcarnitine markers for the diagnosis of other fatty acid oxidation disorders, some patients were misdiagnosed; CACT deficiency was not discovered until these patients were diagnosed using genetic testing, after which the MS/MS results were reviewed and it was found that a few abnormal ratio indices had been ignored.

In the present study, the MS/MS data of 15 patients with CACT deficiency were collected. In addition to establishing typical and atypical acylcarnitine profiles for these patients, it was evaluated whether acylcarnitine ratios may serve as primary markers in the diagnosis of CACT deficiency by comparing the sensitivity and false-positive rate between the

Highlight box

Key findings

- The ratio indices of primary markers can facilitate the diagnosis of CACT deficiency in newborn screening, increasing sensitivity and reducing false-positivity.

What is known and what is new?

- The primary acylcarnitine markers selected in the diagnosis of CACT deficiency are C12, C14, C16, C18, C16:1, C18:1, and C18:2, but not all patients with CACT deficiency have acylcarnitine profiles that match the profile, some patients have been misdiagnosed.
- The ratios of the primary acylcarnitine markers (C16 + C18:1)/C2, C16/C2, C16:1/C3, and C16:1-OH/C3 were significantly higher than reference value of normal newborn in all the patients; these ratios may represent useful markers that can improve the sensitivity and specificity of CACT deficiency screening.

What is the implication, and what should change now?

- The study findings suggest that increased acylcarnitine ratio indices such as (C16 + C18:1)/C2, C16/C2, C16:1/C3, and C16:1-OH/C3 indicate CACT deficiency and can avoid misdiagnosis and reduce false-positivity during newborn screening.

primary acylcarnitine markers and ratios of primary markers levels to those of other acylcarnitines. For certain fatty acid oxidation disorders, such as medium-chain acyl-coenzyme A (CoA) dehydrogenase deficiency (11) and multiple acyl-CoA dehydrogenase deficiency (12,13), the levels of specific metabolic markers are elevated in heterozygous carriers of pathogenic variants. To evaluate whether the primary acylcarnitine markers are abnormal in heterozygous carriers, MS/MS data was analysed of 20 newborns carrying the c.199-10T>G mutation in *SLC25A20* gene and 40 normal controls. We present the following article in accordance with the STARD reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-22-468/rc).

Methods

Study population

This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), and approved by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University (ethics committee batch numbers: 2022ZSLYEC-177, 2022ZSLYEC-414); individual consent for this retrospective analysis was waived. The MS/MS data of 15 patients who were positively diagnosed with CACT deficiency between June 2016 and December 2021 were retrieved from the Department of Paediatrics of the Sixth Affiliated Hospital, Sun Yat-sen University. The dried blood spots (DBSs) of these patients had been collected before or during the acute onset of CACT deficiency, with onset indicators including respiratory and cardiac arrest, coma, cyanosis of the lips, and lethargic reactions. The 15 patients belonged to 14 unrelated families with non-consanguineous marriages in Guangdong Province, and all of the patients died.

The MS/MS data obtained from 28,261 newborn screenings and 53 false-positive samples were retrieved from the Inborn Errors of Metabolism Artificial Intelligence Analysis System 1.0 (the Sixth Affiliated Hospital of Sun Yat-sen University), from January 2020 to December 2021. All infants were younger than 28 days and born in the Guangdong Province. Inclusion criteria for false-positive samples were as follows: (I) among the primary acylcarnitine markers (C14, C16, C18, C16:1, C18:1, and C18:2), three or more indices are higher than the reference values; (II) CACT deficiency was diagnosed based on MS/MS analysis; and (III) the diagnosis had been excluded via genetic testing.

Based on the results of newborn genetic screening, 20 unrelated normal newborns carrying the c.199-10T>G mutation in *SLC25A20* gene and 40 unrelated controls were retrieved. Inclusion criteria for mutation carriers were as follows: (I) only the c.199-10T>G mutant of the *SLC25A20* gene was detected; (II) the absence of any pathogenic mutations, or likely pathogenic mutations or mutations of uncertain significance in the *CPT2* gene. Inclusion criteria for normal controls were as follows: absence of pathogenic mutations, or likely pathogenic mutations or mutations of uncertain significance in *SLC25A20* and *CPT2* gene. The interpretation of sequence variants was based on the "Standards and guidelines for the interpretation of sequence variants" of 2015, developed by the American College of Medical Genetics and Genomics (14). The MS/MS data for 60 DBSs were retrieved from the Inborn Errors of Metabolism Laboratory of the Sixth Affiliated Hospital of Sun Yat-sen University.

Statistical analysis

MS/MS data were analysed as described previously, and all the indices related to CACT deficiency in literature were used as diagnostic indices (6,7). C12, C14, C16, C18, C16:1, C18:1, and C18:2 were included as the primary acylcarnitine markers and (C16 + C18:1)/C2 and (C16 + C18)/C0 were included as the primary ratio markers. These indices were used as the existing diagnostic criteria for CACT deficiency.

The MS/MS results collected in this study were obtained from different years and instruments. Since the reference values of the indices can vary by year or instrument, the data for all reported indices were normalised to reduce this effect of variation; the original concentration value of indices was divided by the corresponding median and then multiplied by the target median. The target median matched the reference values shown in *Tables 1-3*. The reference range was determined using a nonparametric approach, with the 99.5th and 0.5th percentiles identified as the upper and lower reference limits, respectively. SPSS version 26.0 (IBM, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) were used for the data analyses; $P<0.05$ was considered significant.

Results

Acylcarnitine profile in the 15 patients

Fourteen patients had the homozygous c.199-10T>G (rs541208710) pathogenic mutation in the *SLC25A20* gene, and P3 had compound heterozygous mutations with c.199-

Table 1 Acylcarnitine concentration in DBSs of 15 patients with CACT deficiency detected via tandem mass spectrometry

Patient	Sex	Age of screened	Age of onset	Primary acylcarnitine markers (μmol/L)									Interference indices (μmol/L)				
				C0	C12	C14	C16	C18	C16:1	C18:1	C18:2	C16:1-OH*	C8	C10	C14-OH	C16-OH	C18-OH
P1	Male	6H	3D	12.03	0.21*	0.50*	8.53*	2.70*	N	1.73	0.56	0.17*	0.06	0.16	0.02	0.09*	0.02
P2	Male	2D	2D	9.77	0.34*	0.78*	9.49*	1.87*	0.73*	1.37	0.22	0.15*	0.08	0.14	0.02	0.10*	0.02
P3	Male	4D	2D	10.45	0.81*	0.99*	18.53*	3.69*	N	5.56*	0.55	0.15*	0.08	0.13	0.02	0.08*	0.02
P4	Male	4D	2D	7.26*	0.28*	0.90*	12.08*	2.35*	1.44*	2.87*	0.56	0.14*	0.07	0.14	0.02	0.11*	0.03
P5	Female	2M2D	2M1D	3.38*	0.09	0.28	9.94*	0.85	N	1.76	0.40	0.04	0.02	0.02	0.01	0.04	0.01
P6	Female	5D	4D	9.57	0.38*	0.40*	5.04	1.98*	0.43	3.84*	0.98*	0.15*	0.06	0.12	0.02	0.04	0.01
P7	Male	2M26D	2M24D	3.19*	0.09	0.16	3.08	0.63	0.26	1.33	0.23	0.03	0.07	0.29*	0.01	0.04	0.01
P8	Female	1D	1D	2.02*	0.09	0.26	4.60	0.98	0.45	1.53	0.14	0.06	0.03	0.07	0.01	0.05	0.02
P9	Male	1D	5D	12.71	0.73*	1.16*	17.93*	5.80*	1.72*	5.70*	0.94*	0.42*	0.27*	0.72*	0.13*	0.56*	0.13*
P10	Male	1D	1D	6.33*	0.53*	1.02*	11.27*	2.24*	1.06*	3.13*	0.43	0.22*	0.23*	0.50*	0.08*	0.26*	0.06*
P11	Female	2D	2D	7.23*	0.52*	1.11*	14.83*	2.65*	1.47*	4.30*	0.50	0.22*	0.14*	0.37*	0.10*	0.24*	0.08*
P12	Female	3D	4D	12.76	0.08	2.71*	22.51*	5.38*	1.62*	8.38*	0.84*	0.43*	0.21*	0.62*	0.07*	0.15*	0.06*
P13	Male	8D	6D	8.09*	0.58*	0.80*	8.43*	1.62	0.60*	2.68	0.67	0.13*	0.12*	0.58*	0.01	0.03	0.01
P14	Male	4D	1D	8.29*	0.54*	0.71*	12.54*	2.88*	1.28*	6.78*	1.05*	0.20*	0.11*	0.34*	0.03	0.15*	0.04*
P15	Male	2D	1D	10.91	0.58*	1.18*	10.35*	2.87*	1.79*	2.29	0.51	0.20*	0.16*	0.36*	0.05*	0.27*	0.09*
Reference value	–	–	–	9.00–62.00	0.02–0.20	0.03–0.36	0.50–5.75	0.25–1.68	0.02–0.45	0.5–2.75	0.05–0.72	0.01–0.09	0.01–0.10	0.02–0.18	0.00–0.03	0.01–0.07	0.00–0.03

*, indices suggested in this study; *, indicate not within the reference range. DBSs, dried blood spots; CACT, carnitine-acylcarnitine translocase; C0, free carnitine; C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C16, palmitoylcarnitine; C18, stearylcarnitine; C16:1, hexadecenoylcarnitine; C18:1, octadecenoylcarnitine; C18:2, linoleylcarnitine; C16:1-OH, hydroxy hexadecenoylcarnitine; C8, octanoylcarnitine; C10, decanoylcarnitine; C14-OH, 3-hydroxy tetradecanoylcarnitine; C16-OH, 3-hydroxy palmitoylcarnitine; C18-OH, 3-hydroxy stearylcarnitine; H, hours; D, days; N, no detected value; M, months.

Table 2 Ratios of acylcarnitine in DBSs of 15 patients with CACT deficiency

Patient	Primary ratios[1]		Additional ratios[2]					
	(C16 + C18)/C0	(C16 + C18:1)/C2	C14/C3	C16/C2	C16/C3	C18/C3	C16:1/C3	C16:1-OH/C3
P1	0.93	0.89	0.79	0.74	13.54	4.29	N	0.27
P2	1.16	1.18	1.95	1.03	23.73	4.68	1.83	0.38
P3	2.13	1.61	0.96	1.24	17.99	3.58	N	0.15
P4	1.99	1.71	1.50	1.38	20.13	3.92	2.40	0.23
P5	3.19	4.48	1.47	3.81	52.32	4.47	N	0.21
P6	0.73	2.13	1.11	1.21	14.00	5.50	1.19	0.42
P7	1.16	1.29	0.89	0.90	17.11	3.50	1.44	0.17
P8	2.76	5.19	2.00	3.90	35.38	7.54	3.46	0.46
P9	1.87	1.44	0.58	1.09	8.92	2.89	0.86	0.21
P10	2.13	1.94	1.52	1.52	16.82	3.34	1.58	0.33
P11	2.42	2.28	2.41	1.77	32.24	5.76	3.20	0.48
P12	2.19	2.12	4.93	1.54	40.93	9.78	2.95	0.78
P13	1.24	2.45	3.08	1.86	32.42	6.23	2.31	0.50
P14	1.86	2.38	0.86	1.54	15.11	3.47	1.54	0.24
P15	1.21	0.89	0.85	0.73	7.45	2.06	1.29	0.14
Reference value	0.02–0.42	0.09–0.75	0.02–0.34	0.04–0.65	0.30–5.00	0.12–1.50	0.04–0.34	0.01–0.07

[1], indices suggested in the literature (6,7); [2], indices suggested in this study. DBSs, dried blood spots; CACT, carnitine-acylcarnitine translocase; C16, palmitoylcarnitine; C18, stearylcarnitine; C0, free carnitine; C18:1, octadecenoylcarnitine; C2, acetylcarnitine; C14, tetradecanoylcarnitine; C3, propionylcarnitine; C16:1, hexadecenoylcarnitine; C16:1-OH, hydroxy hexadecenoylcarnitine; N, no detected value.

10T>G and c.476T>C. No pathogenic or likely pathogenic mutation was identified in the *CPT2* gene of any of the 15 patients. The DBSs of patients P1, P9, and P12 were collected before the disease onset, whereas those of others were collected during the disease onset. The acylcarnitine concentrations in the 15 patients are shown in *Table 1*. The concentrations of C0 in P1, P9, and P12 were within the normal range; the median concentration was 12.71 μmol/L (12.03–12.76 μmol/L). The other 12 patients showed relatively lower concentrations of C0 during onset; the median concentration was 7.68 μmol/L (2.02–10.91 μmol/L).

Among the 15 patients, 11 exhibited elevated C12 levels, 12 exhibited elevated C14 and C16 levels, 11 exhibited elevated C18 levels, 9 exhibited elevated C16:1 levels, 8 exhibited elevated C18:1 levels, and 4 exhibited elevated C18:2 levels. In addition, the C16:1-OH level was found to be elevated in 12 samples at the same frequency as that of C16 and C14 and at a higher frequency than that of other indices. Through the use of long-chain acylcarnitine (C12, C14, C16, C18, C16:1, C18:1, and C18:2) as the primary diagnostic markers for CACT deficiency, P1 to P6 had the typical acylcarnitine profile of CACT deficiency, showing decreased or normal free carnitine (C0) levels and increased C12, C14, C16, C18, C16:1, C18:1, or C18:2 levels. P7 and P8 showed significantly decreased C0 and normal C12, C14, C16, C18, C16:1, C18:1, and C18:2 levels, which was judged as primary carnitine deficiency. P9 to P15 also showed increased C8, C10, C14-OH, C16-OH, and C18-OH levels as well as increased long-chain acylcarnitine levels, which was judged as multiple acyl-CoA dehydrogenase deficiency or mitochondrial trifunctional protein deficiency.

Acylcarnitine ratios in the 15 patients

The acylcarnitine ratios at levels significantly higher than

Table 3 False-positive rates of indices in newborn screening (n=28,261)

Indices	Positive number	Positive value*	Reference value	False-positive rate
C12	249	0.25±0.05[1]	0.02–0.20[1]	0.88%
C14	46	0.44±0.09[1]	0.03–0.36[1]	0.16%
C16	131	6.46±0.76[1]	0.50–5.75[1]	0.46%
C18	155	1.96±0.26[1]	0.25–1.68[1]	0.55%
C16:1	58	0.51±0.05[1]	0.02–0.45[1]	0.21%
C18:1	145	3.03±0.30[1]	0.5–2.75[1]	0.51%
C18:2	60	0.83±0.13[1]	0.05–0.72[1]	0.21%
C16:1-OH	50	0.12±0.07[1]	0.01–0.09[1]	0.18%
(C16 + C18)/C0	100	0.48±0.05	0.02–0.42	0.35%
(C16 + C18:1)/C2	16	0.96±0.18	0.09–0.75	0.06%
C14/C3	7	0.45±0.09	0.02–0.34	0.02%
C16/C2	6	0.80±0.08	0.04–0.65	0.02%
C16/C3	16	5.53±0.55	0.30–5.00	0.06%
C18/C3	20	1.69±0.19	0.12–1.50	0.07%
C16:1/C3	24	0.39±0.05	0.04–0.34	0.08%
C16:1-OH/C3	22	0.11±0.05	0.01–0.07	0.08%

*, mean values ± standard deviation; [1], the unit is μmol/L. C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C16, palmitoylcarnitine; C18, stearylcarnitine; C16:1, hexadecenoylcarnitine; C18:1, octadecenoylcarnitine; C18:2, linoleylcarnitine; C16:1-OH, hydroxy hexadecenoylcarnitine; C0, free carnitine; C2, acetylcarnitine; C3, propionylcarnitine.

the reference value in all 15 patients are shown in *Table 2*. In addition to the (C16 + C18)/C0 and (C16 + C18:1)/C2 ratios recommended in the present study, the ratios of C14/C3, C16/C2, C16/C3, C18/C3, C16:1/C3, and C16:1-OH/C3 were also significantly increased, and the median levels of these eight ratios were 1.87 μmol/L (0.73–3.19 μmol/L), 1.94 μmol/L (0.89–5.19 μmol/L), 1.47 μmol/L (0.58–4.93 μmol/L), 1.38 μmol/L (0.73–3.9 μmol/L), 17.99 μmol/L (7.45–52.32 μmol/L), 4.29 μmol/L (2.06–9.78 μmol/L), 1.71 μmol/L (0.86–3.46 μmol/L), and 0.27 μmol/L (0.14–0.78 μmol/L), respectively.

False-positive rate

To compare the false-positive rate of the acylcarnitines markers to that of marker ratios, the MS/MS data of 28,261 newborn screening samples were analysed (*Table 3*). For newborn screening, the false-positive rate of acylcarnitine marker use was 0.16% to 0.88% and that of the marker ratios was 0.02% to 0.35%. The false-positive rate of all ratios, except for (C16 + C18)/C0, was less than 0.08%.

Sensitivity of acylcarnitines and ratios

To compare the sensitivity of acylcarnitines *vs.* ratios in the diagnosis of CACT deficiency, the MS/MS data of 15 patients with CACT deficiency and 53 false-positive cases were analysed (*Figure 1*). There was an overlap or partial overlap of all acylcarnitine indices between the positive and false-positive samples, indicating that none of the single acylcarnitines (C12, C14, C16, C18, C16:1, C18:1, C18:2, and C16:1-OH) could separate patients with CACT deficiency from the false-positive samples. However, all ratios were distinguishable between the two groups, particularly (C16 + C18:1)/C2, C16/C2, C16:1/C3, and C16:1-OH/C3.

The levels of acylcarnitines in the carriers

The comparisons of acylcarnitines and ratios between carriers and normal controls are presented in *Table 4*. There was no significant difference in acylcarnitine concentrations between carriers and normal controls (P≥0.05). The (C16

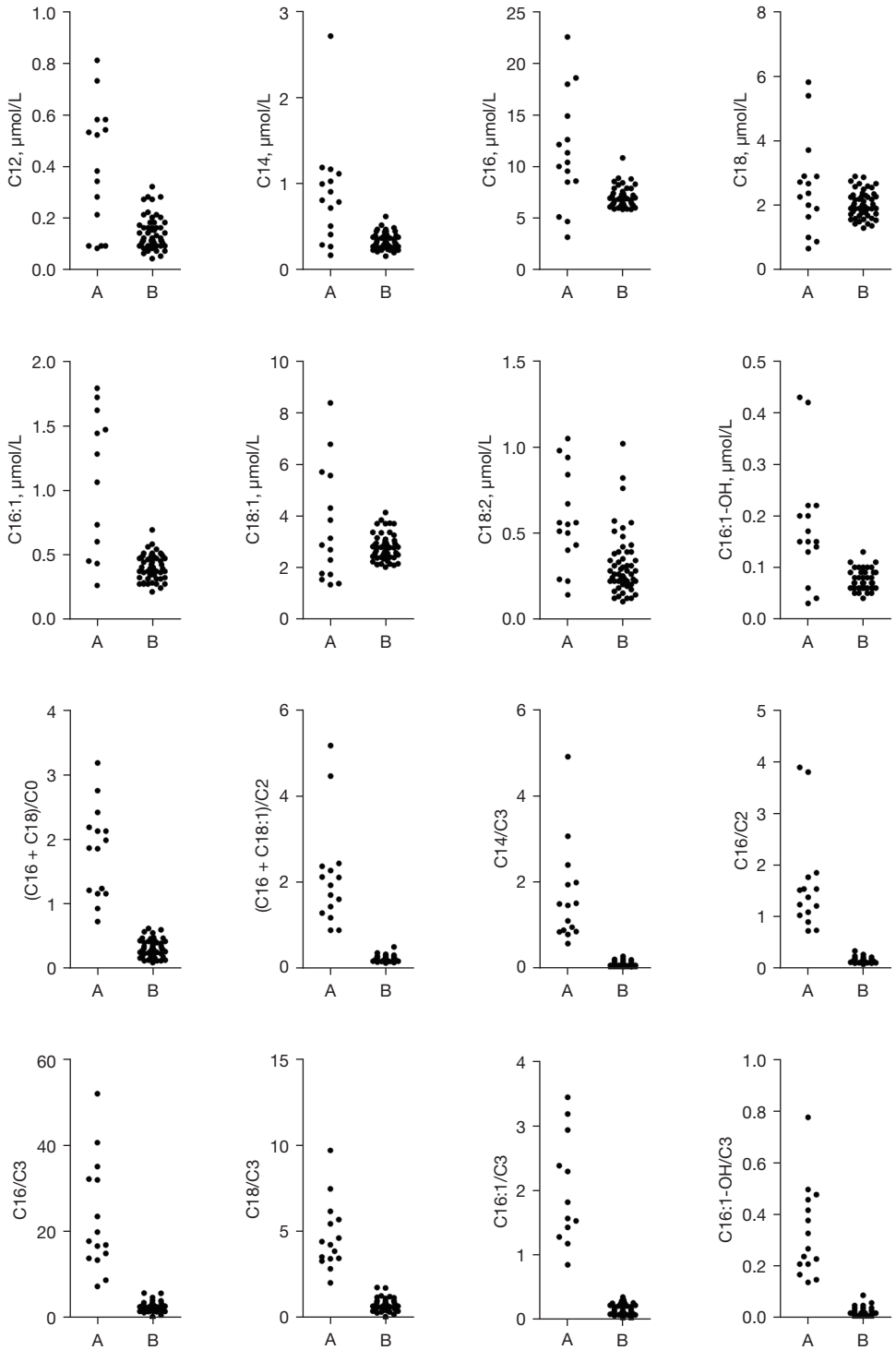

Figure 1 Evaluation of the sensitivity of indices by comparing 15 patients with CACT deficiency and 53 false-positive samples. Group A: 15 patients with CACT deficiency. Group B: 53 false-positive cases. C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C16, palmitoylcarnitine; C18, stearylcarnitine; C16:1, hexadecenoylcarnitine; C18:1, octadecenoylcarnitine; C18:2, linoleylcarnitine; C16:1-OH, hydroxy hexadecenoylcarnitine; C0, free carnitine; C2, acetylcarnitine; C3, propionylcarnitine; CACT, carnitine-acylcarnitine translocase.

Table 4 Comparison of indices in heterozygous carriers and normal controls

Indices	Heterozygous carriers (n=20)	Normal controls (n=40)	P
C12	0.06±0.05[1]	0.07±0.02[1]	0.29
C14	0.15±0.06[1]	0.17±0.04[1]	0.23
C16	2.62±0.76[1]	2.59±0.57[1]	0.90
C18	0.81±0.19[1]	0.79±0.17[1]	0.65
C16:1	0.17±0.08[1]	0.18±0.05[1]	0.81
C18:1	1.45±0.62[1]	1.40±0.30[1]	0.76
C18:2	0.32±0.18[1]	0.23±0.08[1]	0.05
C16:1-OH	0.04±0.01[1]	0.03±0.01[1]	0.06
(C16 + C18)/C0	0.13±0.05	0.15±0.04	0.24
(C16 + C18:1)/C2	0.27±0.08	0.18±0.04	<0.01
C14/C3	0.09±0.04	0.09±0.03	0.89
C16/C2	0.17±0.06	0.12±0.03	<0.01
C16/C3	1.66±0.66	1.43±0.42	0.17
C18/C3	0.51±0.17	0.44±0.14	0.08
C16:1/C3	0.11±0.06	0.10±0.03	0.35
C16:1-OH/C3	0.02±0.007	0.02±0.005	0.01

Heterozygous carriers: only the c.199-10T>G mutant of the *SLC25A20* gene was present without any pathogenic and likely pathogenic mutations and mutations of uncertain significance in the *CPT2* gene. Normal controls: absence of pathogenic and likely pathogenic mutations and mutations of uncertain significance in *SLC25A20* and *CPT2* gene. Data are presented as mean values ± standard deviation. [1], the unit is μmol/L. C12, dodecanoylcarnitine; C14, tetradecanoylcarnitine; C16, palmitoylcarnitine; C18, stearylcarnitine; C16:1, hexadecenoylcarnitine; C18:1, octadecenoylcarnitine; C18:2, linoleylcarnitine; C16:1-OH, hydroxy hexadecenoylcarnitine; C0, free carnitine; C2, acetylcarnitine; C3, propionylcarnitine.

+ C18:1)/C2, C16/C2, and C16:1-OH/C3 ratios were significantly different between the two groups (P<0.05), but the values were within the reference value range of newborn screening.

Discussion

CACT deficiency is a rare and severe mitochondrial fatty acid oxidation disorder. In China, approximately 22 patients with CACT deficiency have been reported in Guangdong (n=9) (9,15), Guangxi (n=4) (16), Shanghai (n=4) (17), Hong Kong (n=3) (10), and Hunan (n=2) (8); the splicing mutation c.199-10T>G is frequently detected in these patients, including homozygous (n=15) and compound heterozygous (n=6) mutations. The c.199-10T>G mutation is a splicing mutation that occurs in a conserved sequence in intron 2 of the *SLC25A20* gene and is likely a founder mutation in the Chinese population, especially in southern China (9,15-19). In the present study, 14 patients had a homozygous mutation c.199-10T>G and the 20 carriers also showed this mutation. Patients with the mutation c.199-10T>G usually present a severe clinical phenotype; similarly, the patients in this study had early onset and two patients died 2 months after birth; all other patients died during the early neonatal period.

Twelve DBSs were collected at the disease onset. The DBSs of P1, P9, and P12 were collected before onset of the disease at 6 hours, day 1, and day 3 postpartum, respectively. P1 had a family history of four older siblings who died of unknown causes during the neonatal period; the clinician assessed that it was necessary to rule out the suspected genetic metabolic disease, so the DBS was collected at the 6th hour postpartum (non-feeding) for MS/MS analysis. P9 had a family history of CACT deficiency, with P4 being the elder brother of P9. Although P4 had been diagnosed with CACT deficiency, the parents refused prenatal diagnosis of P9; therefore, P9 was only tested by MS/MS and gene

sequencing after birth. Although their MS/MS results were analysed as soon as possible, P1 and P9 could not be saved. P12 presented normal at three days postpartum and was screened for neonatal metabolic diseases as the other newborns, but he developed symptoms on the fourth day. MS/MS patient data from before and after onset of the disease were compared and displayed slightly higher C0 levels in the former but no significant difference in long-chain acylcarnitine content. Newborn screening is only performed 72 hours after birth in China, while the onset of CACT deficiency usually occurs 72 hours. While the timing of newborn screening may not be appropriate for CACT deficiency, the results of screening are still important for CACT deficiency diagnosis and is useful in resolving any medical disputes.

The retrospective analysis of MS/MS data from 15 patients showed that the acylcarnitine profiles of CACT deficiency could be classified into three categories. The first category represented a typical profile (P1–P6), showing a decreased or normal C0 level and increased C12, C14, C16, C18, C16:1, C18:1, or C18:2 levels. The second category for patients P7 and P8 showed a significant decrease in the C0 level and a concentration of long-chain acylcarnitines within the normal range. The third category for patients P9–P15 showed the presence of interfering acylcarnitines, including C8, C10, C14-OH, C16-OH, and C18-OH. If the long-chain acylcarnitines (C12, C14, C16, C18, C16:1, C18:1, and C18:2) were used as the primary diagnostic markers for CACT deficiency, the second and third categories may have been misdiagnosed. The MS/MS results of P7 and P8 indicated primary carnitine deficiency and those of P9 to P15 indicated multiple acyl-CoA dehydrogenase deficiencies or mitochondrial trifunctional protein deficiencies. Various fatty acid oxidation disorders can cause secondary carnitine deficiency, which may cause a reduction of other acylcarnitine levels or even of normal acylcarnitine profiles; therefore, the calculated specificity ratios applied to newborn screening can assist in the identification of affected newborns, and the same is true for CACT deficiency. The ratios of the long-chain acylcarnitines comprising (C16 + C18)/C0, (C16 + C18:1)/C2, C14/C3, C16/C2, C16/C3, C18/C3, C16:1/C3, and C16:1-OH/C3 were significantly higher than reference value of normal newborn in all the patients. If the ratios were added to the diagnostic indices in addition to long-chain acylcarnitines, CACT deficiency could be diagnosed based on MS/MS data for the 15 patients. The MS/MS results of primary carnitine deficiency showed that the values of these ratios were within the normal range. However, positive samples of multiple acyl-CoA dehydrogenase deficiency and mitochondrial trifunctional protein deficiency were insufficient to validate the status of these ratios in MS/MS. This constitutes a limitation of this study, and future studies are required to investigate this conclusion.

Exogenous feeding, fasting, and other factors may induce a temporary increase in levels of long-chain acylcarnitines such as C16 and C18, which causes a false-positive result. MS/MS data from 28,261 newborns were analyzed to validate the false-positive results of long-chain acylcarnitines and ratio indices. C16 and C18 were found to have high false-positive rates in newborn screening (at 0.46% and 0.55%, respectively). Except for (C16 + C18)/C0, the false-positive rate of other ratios was lower than that of acylcarnitines indices (0.02–0.08% *vs.* 0.16–0.88%). Furthermore, by comparing patients with CACT deficiency and false-positive cases, none of the single long-chain acylcarnitines were found to be able to separate patients from the false-positive cases; however, all of the ratios showed good discrimination between the two groups. The results of this study suggest that the ratio indices such as (C16 + C18:1)/C2, C16/C2, C16:1/C3, and C16:1-OH/C3 may represent useful markers that can improve the sensitivity and specificity of CACT deficiency screening because of the false-positive rates and specificity. A previous study suggested that the ratio of long-chain acylcarnitines to propionylcarnitine (C3) in newborn screening, including (C16 + C18:1)/C3, C18/C3, C3/C16, C16-OH/C3, and C14/C3, can serve as reliable indices in CPT II deficiency screening, similar to the findings herein (20). Another study suggested that CPT II deficiency can be screened for using (C16 + C18:1)/C2 and C14/C3; the simultaneous increase of only (C16 + C18:1)/C2 and C14/C3 may also detect CACT deficiency (21). These conclusions are consistent with the results of this study. However, this study shows limitations with the lack of data validation of the false-negative rate of this ratio, and further studies with more data are required to prove this hypothesis.

Some reports have showed that the levels of specific metabolic markers are elevated in heterozygous carriers of pathogenic variants for medium-chain acyl-CoA dehydrogenase deficiency, multiple acyl-CoA dehydrogenase deficiency, and similar disorders (11-13). The findings suggest that no significant difference in long-chain acylcarnitine concentrations was observed between heterozygous carriers and normal controls, and the test

values of acylcarnitines in carriers were all within the reference value range of newborn screening; this suggests that carriers of the c.199-10T>G mutation in *SLC25A20* may not exhibit abnormal concentrations of long-chain acylcarnitines.

The concentration of C0 was significantly lower in P8 than in other patients. Besides the homozygous mutation c.199-10T>G, P8 also carried a heterozygous mutation c.760C>T (p. Arg254*) in *SLC22A5*, which is the causative gene of primary carnitine deficiency. Study have shown that nonsense mutations are decidedly associated with severe primary carnitine deficiency and a low plasma C0 level in the neonatal period (22); this suggests that the c.760C>T mutation in *SLC22A5* may be responsible for the markedly low C0 concentration in P8. However, this hypothesis requires further testing with more data. Furthermore, P1 was diagnosed at the 6th hour postpartum (no feeding), and the MS/MS results showed typical carnitine spectrum characteristics, indicating abnormal metabolism of long-chain fatty acids at birth, and the false negatives of CACT deficiency due to homozygous mutation c.199-10T>G are unlikely in newborn screening.

Conclusions

CACT deficiency can be misdiagnosed in newborn screening or diagnosis via MS/MS by relying on primary acylcarnitine markers, such as C12, C14, C16, C18, C16:1, C18:1, and C18:2. Ratios of the long-chain acylcarnitine, such as (C16 + C18:1)/C2, C16/C2, C16:1/C3, and C16:1-OH/C3 may represent useful markers that can improve the diagnosis of CACT deficiency, thereby increasing sensitivity and reducing false-positivity.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Ethics Committee of the Sixth Affiliated Hospital of Sun Yat-sen University (ethics committee batch numbers: 2022ZSLYEC-177, 2022ZSLYEC-414). Due to the retrospective nature of this study, this committee provided an exemption from informed consent.

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