Peer Review File

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**Comment 1:**

Figure 1 and Tables 1, 2

The data of patients screened after onset and those before onset of the disease should be grouped and evaluated separately. Concerning the latter group, why were they screened without symptoms? Perhaps because they had elder symptomatic siblings, I suppose? The only related description in Discussion "the DBSs of P1 and P9 were collected at 6 h and 1 d after birth, respectively, owing to poor family history" sounds unclear.

Reply 1: Thank you for your comment and suggestions. We evaluated the MS/MS data of patients tested before before onset of the disease (3 cases) and after onset of the disease (12 cases). No significant difference was found in acylcarnitine characteristics between the two groups, so grouping was not performed, but the sample collection time and onset time were given in the Table 1 for reference. As for the reasons why P1, P9 and P12 samples were collected before the onset of disease, we added in the second paragraph of discussion.

Changes in the text:

"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 analyzed 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."

Page 12, paragraph 2, lines 214-226.

**Comment 2:**

In the practice of newborn screening for fatty acid oxidation disorders including CACT deficiency and CPT2 deficiency, it gets more difficult to detect affected babies as the day to collect dried blood specimens (DBS) delay. However, there were apparently no definite description concerning when to collect DBS other than a

sentence in Discussion "Newborn screening is performed 72 h after birth". It is preferable to clarify your protocol for newborn screening and evaluate the data of DBS collected within a certain period.

Reply 2: Thank you for this important comment. At present, the newborns' heel blood were collected in 72 hours postpartum for newborn screening in china. The same reference values were used for tandem mass spectrometry results for newborns within 28 days. The tandem mass spectrometry results of one dried blood spots can diagnose 30 to 40 inborn metabolic defects simultaneously, CACT deficiency is one of them. For now, while the findings suggest that 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.

Changes in the text:
"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." Page 13, paragraph 1, lines 226-230.

**Comment 3:** Were there no "true-positive" patients affected by CACT deficiency among 28,261 newborns screened? Did you also rule out CPT2 deficiency, including the three "thermolabile polymorphisms" of CPT2 gene, namely, c.1055T>G (p.F352C), c.1102G>A (p.V368I) and c.1939A>G (p.M647V) ?

Reply 3: Thank you for your suggestion. We know for sure that there were no "true-positive" patients affected by CACT deficiency among 28,261 newborns screened, because another study, for newborn genetic screening, which used 28,261 newborns blood samples for genetic testing, confirmed our results. However, their genetic results include a large number of the three "thermolabile polymorphisms" of CPT2 gene, namely, c.1055T>G (p.F352C), c.1102G>A (p.V368I) and c.1939A>G (p.M647V), homozygous was detected in many samples. A preliminary estimate found that the population carry rates c.1055T>G (p.F352C) was 21.1%, c.1102G>A (p.V368I) was 79.1%, and c.1939A>G (p.M647V) was 8.4% respectively.

**Comment 4:** Inclusion criteria for carriers were as follows: only the c.199-10T>G mutant of the SLC25A20 gene was detected without any pathogenic and likely pathogenic mutations and mutations of uncertain significance in the CPT2 gene. Inclusion criteria for normal controls were as follows: absence of pathogenic and likely pathogenic mutations and mutations of uncertain significance in SLC25A20 and CPT2 gene.

➢ Did you check the three thermolabile polymorphisms mentioned above?

Reply 4: Thank you for your suggestion. Our study didn't take into account these three "thermolabile polymorphisms" of CPT2 gene, c.1055T>G (p.F352C), c.1102G>A (p.V368I) and c.1939A>G (p.M647V). According to the ACMG guidelines, they do not fall into pathogenic, likely pathogenic mutations or mutations of uncertain significance. However, we are very interested in your proposal, and in the future, we will conduct an association study on the three sites and metabolic conditions.

**Comment 5**: Concerning your recommendation of several acylcarnitine ratios as sensitive indices for CACT (and perhaps CPT2) deficiency, you should state the limitation of your study that there are no data of "true-positive" patients included.

Reply 5: We completely agree with your valuable suggestion and have added the explanation of the limitation to the discussion.
Changes in the text:

"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." Page 14, paragraph 1, line251-255.

"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." Page 15, paragraph 1, line273-275.