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# Thermolabile phenotype of carnitine palmitoyltransferase II variations as a predisposing factor for influenza-associated encephalopathy

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**Abstract To assess the etiology of influenza-associated encephalopathy (IAE), a surveillance effort was conducted during 2000–2003 in South-West Japan. All fatal and handicapped patients except one (4/34 patients) exhibited a disorder of mitochondrial β-oxidation evoked by the inactivated carnitine palmitoyltransferase II (CPT II) with transiently elevated serum acylcarnitine ratios ($C_{16:0} + C_{18:1})/C_2 > 0.09$ during high-grade fever. Analyses of genotypes and allele compositions of CPT II revealed a thermolabile phenotype of compound heterozygotes for [1055T > G/F352C] and [1102G > A/V368I], which shows a higher frequency in IAE patients than healthy volunteers ($P < 0.025$). The thermolabile phenotype of CPT II variations may be a principal genetic background of IAE in Japanese.**


*Keywords:* Carnitine palmitoyltransferase II; DNA sequence variation; Influenza-associated encephalopathy; Thermal inactivation

## 1. Introduction

Influenza-associated encephalopathy (IAE) is a severe neurologic complication of influenza infection which is known to be distinct from Reye's syndrome [1,2]. The number of cases of IAE has increased in recent years, with more than 100 children aged below 5–6 years dying annually from IAE in Japan [3,4]. IAE results in high morbidity and mortality and is characterized by a high-grade fever accompanied within 12–48 h by febrile convulsions, often leading to coma and multiple-organ failure. Because the frequency of IAE is higher in Japanese than it is in Caucasians, it is possible that genetic factors play an important role in the aetiology of IAE.

During the last four influenza seasons of 2000–2003, we conducted a survey to investigate the etiology and constitutional predispositions in patients susceptible IAE in South-West Japan. We found that almost all fatal or handicapped IAE patients exhibited transiently elevated the serum levels of long-chain acylcarnitines during high-grade fever >40 °C. The result suggests that the high-risk patients have thermolabile genetic backgrounds of enzymes in the long-chain fatty acid metabolism. In the present study, we report thermolabile carnitine palmitoyltransferase II (CPT II) variation, predominantly found in Japanese IAE children, and discuss the etiology of IAE as a 'thermolabile phenotype of polymorphic variation'.

## 2. Materials and methods

### 2.1. Patients

This investigation was approved by the ethics review committee for human genome analysis at our institution. All participants granted their written informed consent. Surveillance for IAE was conducted during the influenza seasons of 2000 through 2003 in South-West Japan, and a total of 34 patients were diagnosed as having IAE. The diagnosis of IAE was made according to the clinical signs of the disease [3]. All 34 patients had viral antigen, the abrupt onset of seizure and coma that occur within 12–48 h after beginning of a high-grade fever, although these patients did not have previous episodes. One patient (patient #21) had ingested dichophenac sodium at the onset of fever and later died, but fatty degeneration in the liver, a typical pathological finding of Reye's syndrome [5,6], was not observed. Thirteen IAE patients, four familial relations of patient #21 and 79 healthy volunteers agreed to undergo the genome analyses.

### 2.2. Clinical data analyses

EDTA-treated peripheral blood, urine and specimens from throat swabs were obtained from the patients. Profiles of organic acids in urine and acylcarnitines in serum were analyzed by gas chromatography–mass spectrometry [7] (Shimazu Qp5000 Model, Shimazu, Kyoto, Japan) and electrospray tandem mass spectrometry [8] (TSQ7000 Model, Thermo-Quest, Tokyo, Japan), respectively. Influenza virus antigen was detected by enzyme-linked immunosorbent assay (Becton–Dickinson) in specimens from throat swabs.

### 2.3. Assay of CPT II activity

CPT II activities were measured in the homogenates of liver biopsies and COS-7 cells transfected with wild-type (WT) and polymorphic variant CPT II cDNAs in the presence of 1% Tween 20 in the reaction mixture, by detecting of the palmitoyl-L-[methyl-$^3$H]carnitine formed from L-[methyl-$^3$H] carnitine and palmitoyl-CoA [9]. For the analysis of the heat stability of WT and variants of CPT II, the activities of liver and cellular homogenates were measured after incubating the samples at 37 and 41 °C.

### 2.4. Analysis of genomic CPT II

Genomic DNA from whole blood was purified as previously described [10]. PCR of five exons of the *CPT II* gene was carried out with intron-based primers (Table 1) in genomic DNA. For haplotype analysis the CPT II exon 4 region was cloned into pCR®2.1

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Table 1
Exon primers used for PCR amplification of the *CPT2* gene; MA or MB primer used for variant induction

| Region | Forward primer | Reverse primer | Product size (bp) |
|---|---|---|---|
| Exon 1 | cttgtgtttagactccagaactcc | gtcatgagtgactgcagtcaggttg | 292 |
| Exon 2 | ctgtcagccttacactgaccc | aactctcggggcttggtc | 305 |
| Exon 3 | tttagggctatgctgttggg | aggaagggaggatgagacgt | 358 |
| Exon 4-1 | ctctggaggttgatgccatt | acccaagcactgaggacaag | 1472 |
| Exon 4-2 | tagagttcagtgggtagctggct | atccaggcacatctgaagtac | 401 |
| Exon 5 | tttcctgaggtccttttccatcctg | atgaggaagtgatggtagcttttca | 425 |
| MA-V352I | ggcacaaaccgctggtgtgataaat | atttatcacaccagcggtttgtgc | |
| MB-V368I | ctactgccgtccactttagcactcttg | caagagtgctcaaagtggacggcagtag | |

vector (Invitrogen). The sequences of the PCR products and cloned *CPT II* gene were analyzed with the ABI DyeDeoxy Terminator Cycle Sequencing Kit on an ABI-PRISM 3100 Genetic Analyzer (PE-Applied Biosystems). Each PCR product was sequenced in both strands, and the analysis was performed at least twice independently.

### 2.5. Expression of WT and variant CPT IIs in COS-7 cells

A full-length WT CPT II cDNA clone (pCMV6-WT) containing the entire coding region of human CPT II was a gift of V. Esser, the University of Texas. Plasmid pCNV6-WT was used as a parental vector to generate three full-length variant CPT II cDNA clones, pCMV6-MA [1055T > G/F352C], pCMV6-MB [1102G > A/V368I] and pCMV6-MA + B [1055T > G/F352C] + [1102G > A/V368I] by means of a QuickChange® site-directed mutagenesis Kit (Stratagene). The primers used for variant induction are listed in Table 1. The substitutions and integrity of the CPT II cDNAs were confirmed by sequence analysis. pSVβ-Galactosidase control vector (Promega) was co-transfected with various pCMV-6-CPT II plasmids as an internal standard for the monitoring of transfection efficiency. Mock transfection was also carried out as control. After transfection for 72 h, COS-7 cells were washed twice with saline and CPT II activities of WT and variants were analyzed.

## 3. Results

### 3.1. Patients and acylcarnitine ratios

Thirty-four patients (22 male and 12 female) with no underlying disease, ranging in age from 0 to 16 years, with a mean age of 4.7 ± 2.8 years, had IAE. Influenza A, B and A + B virus antigen were detected in nasopharyngeal swabs at 91.2%, 5.9% and 2.9%, respectively. A single patient (patient #21, which was one of the fatal cases) used dichrophenac during the influenza episode.

Laboratory tests of patient serum revealed that 41.2% of the IAE patients exhibited characteristic elevations of serum acylcarnitine ratios [11] $(C_{16:0} + C_{18:1})/C_2 > 0.048$ an upper cutoff value [8,11] (Fig. 1). In particular, over half the patients in the severe IAE group (seven patients), i.e., those with the ratios >0.09, turned to fatal (three female) and handicapped (one male) outcomes. These data indicate that patients in the high-risk group have a marked disorder(s) of mitochondrial long-chain fatty acid metabolism. The most common inborn errors of mitochondrial fatty acid β-oxidation and related metabolism in Japanese are due to CPT II deficiency, with an accumulation of long-chain acylcarnitine in serum and glutaric aciduria type 2 (GA2) at 26.6% and 21.9%, respectively [12]; however, the frequency of these are relatively low at 11% and 5.5%, respectively, in Caucasians, who have the most common deficiency of medium-chain acyl-CoA dehydrogenase (MCAD), which occurs at about 36.6% [13]. The $(C_{16:0} + C_{18:1})/C_2$ ratios of all IAE patients tested, however, normalized or decreased to the borderline ratios between

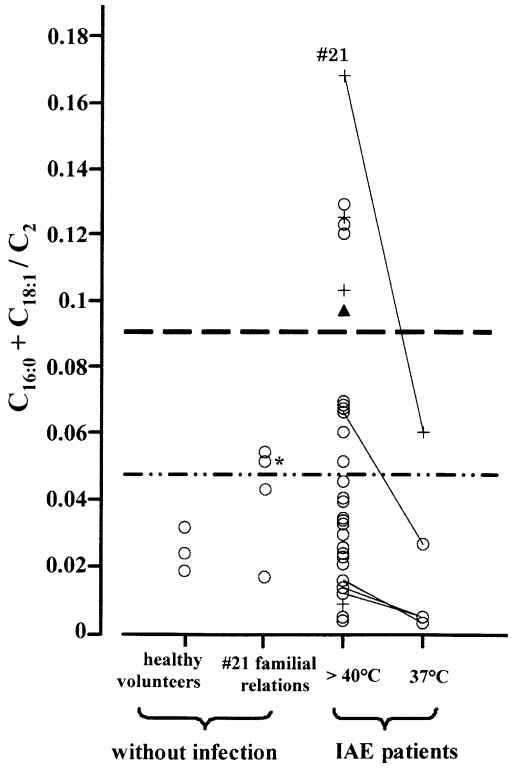


Fig. 1. Distribution of $(C_{16:0} + C_{18:1})/C_2$ ratios of the patients with IAE at the time of high-grade fever/convulsion and normal temperature conditions. The acylcarnitine ratios of patients suffering from IAE, and the family members of patient #21 and one volunteer without infection were analyzed. Upper cut-off range = 0.048 and high-risk patient range = 0.09 are indicated by the thin dashed and bold dashed lines, respectively. (+) Fatal; (▲) handicapped; (*) brother, who had the same genotype as in patient #21.

0.048 and 0.06 at normal temperature after febrile convulsion. These results suggest that the ratios are transiently elevated during febrile convulsions at >40 °C. Familial relations except the mother of patient #21, who carried thermolabile phenotype of CPT II, as described below, exhibited borderline ratios between 0.042 and 0.054 under normal temperature, and a brother who had identical alleles with patient #21, exhibited the ratio of 0.051. Two patients in the high-risk group with ratios >0.09 recovered after infection for 3–4 weeks without any sequelae.

There was one fatal IAE patient (patient #16) with the ratio of 0.004 (Fig. 1), who was diagnosed GA2 based upon an abnormal urinary organic acid profile (data not shown), a disorder of electron transfer in mitochondria. All the other IAE patients with the ratios <0.09 recovered without any severe sequelae. Octanoylcarnitine, a diagnostic marker of MCAD

deficiency [14] was not elevated in any of the Japanese IAE patients so far.

### 3.2. Thermolability of CPT II variants

To further study the temperature dependence of the acylcarnitine levels shown in Fig. 1, liver biopsies were obtained from patient #21 and from one control patient. The specific activity of CPT II of patient #21, who had the highest acylcarnitine ratio of 0.168, was 0.4 ± 0.06 nmol/min/mg protein, being about 36% normal control (1.1 ± 0.3 nmol/min/mg protein, $n = 6$) at 37 °C. It is noteworthy that this patient's CPT II was extremely thermolabile and the specific activity was reduced to about 50% after incubation for 120 min at 41 °C, although control CPT II was reduced only slightly, to 91.4%, under the same assay conditions (Fig. 2). In order to analyze the etiology of the thermolability of the patient's CPT II, we analyzed the genotypes of patient #21 and her familial relations. Sequence analyses of the *CPT II* gene revealed that the patient possessed compound heterozygous variations, i.e., [1055T > G/F352C] + [1102G > A/V368I], and no other reported CPT II mutations and polymorphisms [15] were detected. The F352C substitution has been reported only in Japanese and not in Caucasians to date, and the V368I polymorphic variation is found in both races but has relatively mildly deleterious effects related to CPT II deficiency [15,16]. Her brother also had heterozygous and the father had homozygous compound variations for [1055T > G/F352C] + [1102G > A/V368I]. The sister had heterozygous variation for [1055T > G/F352C] + homozygous variation for [1102G > A/V368I] and the mother had only heterozygous variation for [1102G > A/V368I]. Although a polymorphism [1939A > G/M647V] of CPT II reported [15] was not found in patient #21 and her familial relations, it was found in several IAE patients tested, but no significant difference was observed in the frequency between IAE patients and healthy volunteers (Table 2).

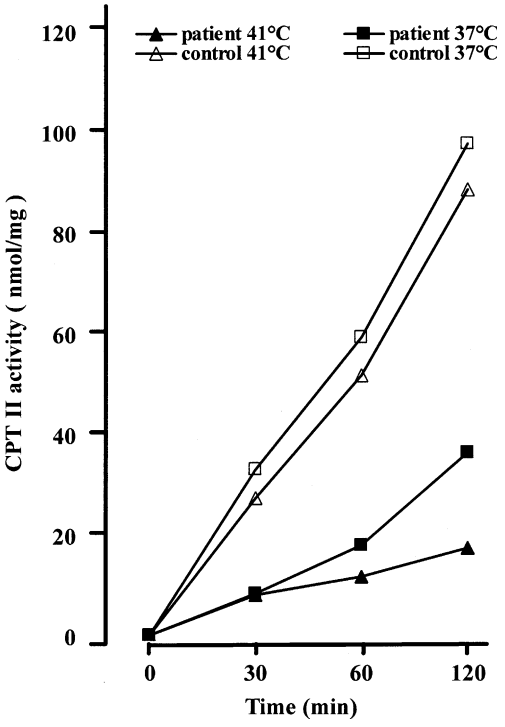


Fig. 2. Time courses of change in the activities of CTP II from the liver homogenates of control and patient #21 at 37 and 41 °C. Data are presented as means ± S.D. ($n = 6$).

Haplotype analysis of the pedigree of patient #21 and the levels of acylcarnitine ratios of her familial relations revealed that haplotype CIM was commonly observed among patient #21 and her relations (father, brother and sister), who exhibited the borderline acylcarnitine ratios at normal temperature (Fig. 3). During febrile convulsions at >40 °C, acylcarnitine level of patient #21, who had the same alleles as in the brother, may increase to the high-risk ratios >0.09. The mother of patient #21 without haplotype CIM exhibited normal acylcarnitine ratio.

Genotypes and allele compositions of CPT II between IAE patients and healthy volunteers are shown in Table 2. Among nine genotypes observed, the frequency of type 9 (FVM-CIM alleles), being identical to the genotype of patient #21, was significantly higher than that in healthy volunteers ($P < 0.025$). There was no other variation with a significantly higher frequency than that found in healthy volunteers has been identified in IEA patients to date, perhaps because of the limited number of patients analyzed.

### 3.3. Expression of F352C and V368I CPT II polymorphic variants and their thermal instability

For in vitro expression of WT and the variant CPT II cDNAs, four cDNAs were overexpressed in COS-7 cells (Fig. 4): pCMV6-WT containing WT CPT II (FVM-CPT II);

Table 2
Genotypes and allele compositions of CPT II between IAE patients and healthy volunteers

| Genotypes | Patients | | Healthy volunteers | | Alleles | *P* |
|---|---|---|---|---|---|---|
| | *n* | Frequency (%) | *n* | Frequency (%) | | |
| Type 1 (F352F-V368V-M647M) | 0 | 0 | 11 | 13.9 | FVM-FVM | |
| Type 2 (F352F-I368I-V647V) | 0 | 0 | 1 | 1.2 | FIV-FIV | |
| Type 3 (F352F-V368I-M647V) | 1 | 7.7 | 3 | 3.8 | FVM*-FIV* | |
| Type 4 (F352F-I368I-M647V) | 1 | 7.7 | 1 | 1.2 | FIM-FIV | |
| Type 5 (F352F-I368I-M647M) | 3 | 23.1 | 25 | 31.6 | FIM-FIM | |
| Type 6 (F352F-V368I-M647M) | 2 | 15.4 | 27 | 34.2 | FVM-FIM | |
| Type 7 (C352C-I368I-M647M) | 1 | 7.7 | 5 | 6.3 | CIM-CIM | |
| Type 8 (F352C-I368I-M647M) | 1 | 7.7 | 0 | 0 | FIM-CIM | |
| Type 9 (F352C-V368I-M647M) | 4 | 30.8 | 6 | 7.6 | FVM-CIM | <0.025 |
| Total | 13 | | 79 | | | |

*Haplotypes not determined.

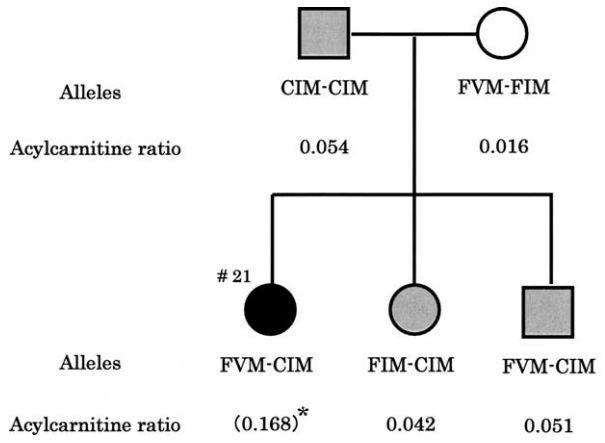


Fig. 3. Haplotype analysis of the pedigree of patient #21 and levels of acylcarnitine ratios of the familial relations. Haplotypes of *CPT II* gene (FVM, FIM and CIM) are illustrated. Serum aclycarnitine ratios at normal temperature (father, mother, sister and brother) and acylcarnitine ratio of patient #21 during febrile convulsions at >40 °C (*). (black) Acylcarnitine ratios >0.09; (gray) borderline acylcarnitine ratios; (white) normal acylcarnitine ratios.

pCMV6-MA containing the [1055T > G/F352C] (CVM-CPT II); pCMV6-MB containing the [1102G > A/V368I] (FIM-CPT II); and pCMV6-MA + B containing the [1055T > G/F352C] + [1102G > A/V368I] (CIM-CPT II). The CPT II activity of vector alone was 0.27 ± 0.03 nmol/min/mg protein ($n$ = 5), being consistent with endogenous enzyme activity, and all of the following data presented have the value of CPT II activity of the vector alone subtracted. The CPT II activity of the COS-7 cells overexpressed with pCMV6-WT was 0.46 ± 0.07 nmol/min/mg protein. The CPT II activities of the CVM-CPT II, FIM-CPT II and CIM-CPT II exhibited as 62.8 ± 7.2%, 102.5 ± 19.6% and 34.7 ± 1.3% of the WT FVM-CPT II activity, respectively, at 37 °C. Next we analyzed WT and the variant CPT II activities under heat stress conditions at 41 °C. Although the activity of WT FVM-CPT II was slightly decreased to 91% at 41 °C during incubation for 120 min, those of FIM-, CVM- and CIM-CPT II were decreased to 91%, 48% and 72%, respectively, at 41 °C, in comparison with those at 37 °C. Among these variants, the enzyme activity of both CIM- and CVM-CPT II was significantly reduced to about 25–30% of WT FVM-CPT II activity at 37 °C. These data support the inactivation in CPT II activity seen in patient #21 at 41 °C in Fig. 2, although these results are from the homozygous changes of [1055T > G] and [1102G > A] alleles of CPT II.

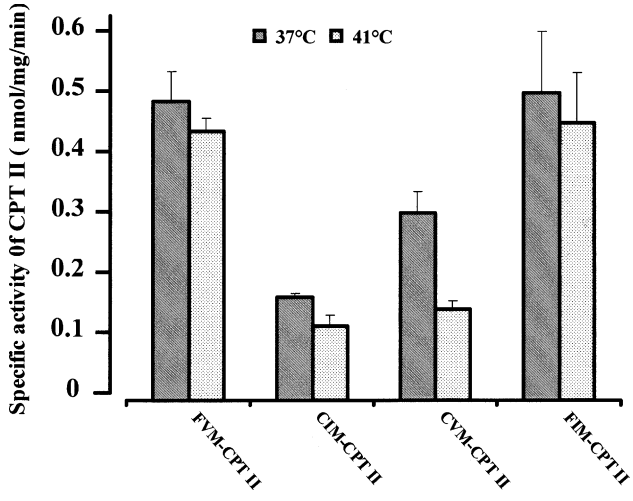


Fig. 4. Comparison of the activities of WT and CTP II variants overexpressed in COS-7 cells at 37 and 41 °C. CPT II activities of WT (FVM-CPT II), the [1055T > G/F352C] (CVM-CPT II), the [1102G > A/V368I] (FIM-CPT II) and the [1055T > G/F352C] + [1102G > A/V368I] (CIM-CPT II) were measured at 37 and 41 °C. Data are presented as means ± S.D. ($n$ = 5).

## 4. Discussion

Encephalitis/encephalopathy is a commonly encountered pediatric disorder in various clinical forms. Among these, IAE has been reported as a complication of influenza, and is of particularly high frequency in Japanese children [3]. In the present study, we found that 41.2% of IAE patients exhibit an elevated serum acylcarnitine ratio – $(C_{16:0} + C_{18:1})/C_2$ > the upper cutoff value of 0.048 – during febrile convulsion and 57% of the high-risk patients with ratios of >0.09 took a clinical turn towards death or severe sequelae. This ratio is a marker of long-chain fatty acid metabolism disorder and the most common disorder of this sort in Japan is due to a CPT II deficiency [12]. In the studied group there was one fatal GA2, disorder of electron transfer in mitochondria, the patient having the value <0.048. These results indicate that all the severe IAE patients who took a clinical turn to fatality (four patients) and severe sequelae (one patient) had disorders of mitochondrial energy metabolism. It is particularly noteworthy that elevated acylcarnitine ratios at the time of high-grade fever were then significantly decreased under a return to normal temperature in all of the follow-up patients. These findings suggest that a continuous high-grade fever, often accompanied by fasting, evokes a systemic and metabolic energy crisis, particularly in the patient with thermolabile polymorphic variations or a deficiency of energy metabolizing enzymes.

The children having the most common inborn errors of mitochondrial fatty acid β-oxidation, such as CPT II deficiency and GA2 in Japanese with the approximate frequencies of 1/100000 [12,17], suffer severe acute encephalopathy and multiple-organ failure (Reye's-like syndrome) without infection. Our present data indicate that severe IAE patients who exhibit thermolabile CPT II are the carriers of haplotype CIM. The allelic frequency of F352C in the Japanese population reported is 0.21 but the variation has not been reported among Caucasian populations [16]. The allelic frequencies of V368I and M647V are 0.70 and 0.04, respectively, in the Japanese population [16] and 0.51 and 0.25, respectively, in Southern European populations [18].

In the present study, we found thermolabile phenotype of CPT II variations in IAE patients, and propose these variations as 'susceptibility variants of IAE'. CPT II, however, is not a rate-limiting enzyme of the metabolic pathway of β-oxidation and when CPT II activities are above 30% those of control, fatty acid oxidation is usually found within the normal range [15]. Under heat stress conditions at 41 °C, the CPT II activities of patient #21 and the transfected CIM- and CVM-CPT II were below 30% of WT CPT II at 37 °C. In regard to the three polymorphic variations of CPT II, such as

F352C, V368I and M647V, 9 genotypes out of 27 expected genotypes were observed in the IAE patients and healthy volunteers, as shown in Table 2. Among these, difference in the frequencies of type 9 (F352C-V368I-M647M) in IAE patients was significantly high between the two groups. Although type 7 (C352C-I368I-M647M) showed the lowest enzyme activity in Fig. 4, differences in the frequencies of type 7 between IAE patients and healthy volunteers were not significant, probably because of the limited number of IAE patients analyzed. The CIM haplotype, as measured in COS-7 cells, was moderately temperature sensitive, while the CPT II in the liver from patient #21 showed more drastic temperature sensitivity. These results suggest that, in addition to the heat-stress by temperature, the metabolic stress by increased fatty acid oxidation flux during fever and fasting may contribute significantly, and/or an involvement of variations of another energy metabolizing enzymes which synergistically induce energy crisis in combination with CPT II variations. Although the molecular mechanisms of the encephalopathy and acute brain edema seen in IAE patients with a disorder of mitochondrial β-oxidation have yet to be clarified, the accumulation of mini-plasmin in the cerebral capillaries in mice with a congenital or acquired abnormality of mitochondrial β-oxidation, and the resulting proteolytical destruction of the blood–brain barrier after influenza virus infection [19], might prove to be one of the related etiologic factors.

Considering the energy metabolism disorder of IAE patients with thermolabile phenotype of CPT II polymorphic variations, hypothermia therapy, administration of L-carnitine for activation of long-chain fatty acid β-oxidation, and an administration of glucose to increase the rate of the citric acid cycle, might prove to be highly therapeutic when confronted with cases of IAE.

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