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A Heterozygous Missense Mutation in Adolescent-Onset Very Long-Chain Acyl-CoA Dehydrogenase Deficiency with Exercise-Induced Rhabdomyolysis

Shin Hisahara,[1] Takashi Matsushita,[1] Hiroyasu Furuyama,[2] Go Tajima,[3] Yosuke Shigematsu,[4] Tomihiro Imai[1,5] and Shun Shimohama[1]

[1]Department of Neurology, School of Medicine, Sapporo Medical University, Sapporo, Hokkaido, Japan
[2]Department of Neurology, Sapporo Yamanoue Hospital, Sapporo, Hokkaido, Japan
[3]Department of Pediatrics, Hiroshima University Graduate School of Biomedical Sciences & Health Sciences, Hiroshima, Hiroshima, Japan
[4]Department of Health Science, Faculty of Medical Sciences, University of Fukui, Fukui, Fukui, Japan
[5]Department of Occupational Therapy, School of Health Sciences, Sapporo Medical University, Sapporo, Hokkaido, Japan

Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency is characterized by impaired mitochondrial *β*-oxidation of fatty acids. The fatty acid oxidation plays a significant role in energy production especially in skeletal muscle. VLCAD is one of four acyl-CoA dehydrogenases with different-chain length specificity and catalyzes the initial step in mitochondrial *β*-oxidation of fatty acids. While the clinical phenotypes in neonates and infants are described as severe, adolescent-onset or adult-onset VLCAD deficiency has a more benign course with only skeletal muscle involvement. These myopathic phenotypes are characterized by episodic muscle weakness and rhabdomyolysis triggered by fasting and strenuous exercise. We report a male teenager who manifested repeated episodes of rhabdomyolysis immediately after exertional exercise. Rhabdomyolysis was diagnosed based on the marked elevation of serum creatine kinase and myoglobinuria. Acylcarnitine analysis by tandem mass spectrometry (MS/MS) revealed elevation of serum tetradecenoylcarnitine (C14:1-AC), which represents an abnormal acylcarnitine profile associated with the mitochondrial *β*-oxidation defect. High performance liquid chromatographic analysis showed decreased production of 2-hexadecenoyl-CoA (C16:1) from palmitoyl-CoA (C16:0), indicating the defect of VLCAD activity. Direct sequencing of the *acyl-CoA dehydrogenase, very long-chain* gene (*ACADVL*) that codes VLCAD revealed a heterozygous mutation (c.1242G>C) in exon 12 (E414D), which is a novel mutation in myopathic-type VLCAD deficiency. Because VLCAD functions as a homodimer, we assume that this heterozygous mutation may exhibit dominant-negative effect. This patient remains asymptomatic thereafter by avoiding exertional exercise. The findings of reduction of enzyme activity and clinical features associated with this novel missense mutation of VLCAD are discussed.

Keywords: *acyl-CoA dehydrogenase, very long-chain* gene; heterozygous missense mutation; rhabdomyolysis; tandem mass spectrometry; very long-chain acyl-CoA dehydrogenase deficiency
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Introduction

Free fatty acids are converted into acyl-CoA and used for mitochondrial metabolism via fatty acid oxidation. Acyl-CoA is transported to the inner mitochondrial membrane, the site of *β*-oxidation. Mitochondrial *β*-oxidation of fatty acids plays a pivotal role in energy production especially in skeletal muscle (Bartlett and Eaton 2004). To cross the impermeable outer mitochondrial membrane, acyl-CoAs are converted into acylcarnitine. For the initial step of fatty acid *β*-oxidation within mitochondria, fatty acid oxidation occurs via a series of catabolic reactions that progressively shorten acyl-CoA by two carbons during each reaction cycle to produce acetyl-CoA. The four specific acyl-CoA dehydrogenases with carbon chain length specificities are involved. Thus, enzymatic insufficiency results in abnormal profile of various carbon length acyl groups. Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency is an inborn error of fatty acid *β*-oxidation in mitochondria. VLCAD is a homodimer of 70-kDa subunits, and

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Correspondence: Shin Hisahara, M.D., Ph.D., Department of Neurology, School of Medicine, Sapporo Medical University, South 1, West 17, Chuo-ku, Sapporo, Hokkaido 060-8556, Japan.
e-mail: hisahara@sapmed.ac.jp

is localized in the inner mitochondrial membrane and catalyzes the introduction of Δ2,3-double bond into the straight chain fatty acyl-CoA with chain length of 14-20 carbons (Aoyama et al. 1994).

VLCAD deficiency has a broad clinical spectrum. The most severe form manifests neonatal cardiomyopathy, arrhythmia, liver dysfunction, and is generally fatal in the first year of life. An infantile form presents clinically as episodes of hypoketotic hypoglycemia in early childhood. However, the most common form is characterized by adolescent- or adult-onset myopathy with myalgia, cramps, and rhabdomyolysis. In late childhood and early adulthood, episodic myalgia and frank rhabdomyolysis predominate, and these symptoms extend into adulthood, often with weakness. Cardiomyopathy and liver dysfunction are not found in adult-onset VLCAD deficiency (Arnold et al. 2009; Wilcken 2010).

To screen for VLCAD deficiency, tandem mass spectrometry (MS/MS) can be used to analyze serum specimens to detect elevated levels of tetradecenoylcarnitine (C14:1-AC) (Tajima et al. 2008). To confirm the diagnosis, further analysis using high performance liquid chromatography (HPLC) can be used to demonstrate decreased 2-hexadecenoyl-CoA production in lymphocytes, which indicates impaired VLCAD activity (Shigematsu et al. 2002; Tajima et al. 2008). Thus, the enzymatic assay is useful for the precise diagnosis of VLCAD deficiency.

The genetic basis of VLCAD deficiency is complex. Previous studies have identified approximately one hundred mutations of the *acyl-CoA dehydrogenase, very long-chain* gene (*ACADVL*) (Gregersen et al. 2008). Among 55 families with VLCAD deficiency, patients with severe neonatal phenotype had mutations that result in no residual VLCAD activity, whereas those with milder childhood and adult phenotypes had mutations with residual enzyme activity (Andresen et al. 1999). These results strongly indicate a clear correlation between genotype and disease severity. Basically, VLCAD deficiency is inherited in an autosomal recessive manner. Homozygous or compound heterozygous mutations were identified in 94 of 110 alleles in 55 families of VLCAD deficiency (Andresen et al. 1999). However, several studies have also shown single allele mutations in *ACADVL*, indicating that even partial defect of VLCAD activity may result in clinical manifestations, even if mild (Andresen et al. 1999; Mathur et al. 1999; Pons et al. 2000; Schymik et al. 2006; Laforet et al. 2009).

In this report, we describe a case of adolescent-onset VLCAD deficiency. This patient presented with relatively severe exercise-induced rhabdomyolysis, but was able to remain asymptomatic thereafter by avoiding exertional exercise. We demonstrated not only decreased VLCAD enzyme activity, but also a novel heterozygous missense mutation together with multiple short genetic variations.

Case Presentation

A 17-year-old man was admitted because of severe myalgia and muscle weakness during and after exercise. One year before admission, he first noticed severe bilateral thigh and calf pain as well as muscle cramp near the finish of a 17-km run during a marathon meet in his high school. Although he ceased running immediately, he experienced aggravating myalgia and muscle weakness and passed cola-colored urine in the evening. He was taken to a hospital and rhabdomyolysis was diagnosed based on marked elevation of serum creatine kinase (CK) to 305,100 U/L and myoglobinuria. Eight months before admission, he again experienced weakness in bilateral lower extremities during ski exercise. He was transported to a local hospital and his CK had increased to 40,000 U/L. Two months before admission, he had bilateral calf and thigh pain after a 5-km walk. Further investigations of causative factors and underlying etiology of repetitive exercise-induced rhabdomyolysis were required.

The patient was the first child of non-consanguineous healthy parents. He was born at 40 weeks of gestation with a birth weight of 3,140 g, and he attained psychomotor and growth developmental milestones normally. On admission to our hospital, he was 179 cm in height and weighed 76 kg. He was not taking medications such as hypolipidemic agents and diuretics, and was not using nutritional supplements. Physical and neurological examinations showed no abnormalities. There was no evidence of cardiomyopathy, hepatosplenomegaly, and hypoglycemia. Computed tomography revealed no prominent muscular atrophy and no fatty degeneration. Serum levels of CK, aspartate aminotransferase and alanine aminotransferase were normal, but both total and free carnitine levels were obviously low [21.4 μmol/L (normal range 45-91) and 16.7 μmol/L (36-74), respectively]. Acylcarnitine analysis of serum sample was performed by tandem mass spectrometry (MS/MS) and revealed elevation in C14:1-AC [0.44 nmol/ml (< 0.4)] (Fig. 1). Enzymatic assay for VLCAD activity by measuring 2-hexadecenoyl-CoA production in lymphocytes showed a marked decrease in VLCAD activity [3.48 (55.3 ± 18.1) pmol/min/10^6/cells], which confirmed a diagnosis of VLCAD deficiency (Tajima et al. 2008). The biochemical profile of this patient was reported previously [Patient No. 6 in (Tajima et al. 2008)].

We performed direct genome sequencing of 6775 base pairs (bp) including all 20 exons of *ACADVL* (NT_010718.16; 6725713 to 6732487). Primer design was developed to create upstream and downstream of each exon for forward and reverse primers, respectively. Sanger sequencing was processed using a DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare) and an ABI PRISM3130 automated sequencer. This region contains a part of 5′-untranslated region (UTR) of the next gene *DLG4*, 5′- and 3′-UTR of *ACADVL*, and a part of 3′-UTR of the next gene *DVL2*. We found a novel heterozygous mutation c.1242G>C (p.E414D) in exon 12 (Fig. 2). Using data from the 1000 Genome project (http://browser.1000genomes.org/index.html, dbSNP build 142, latest

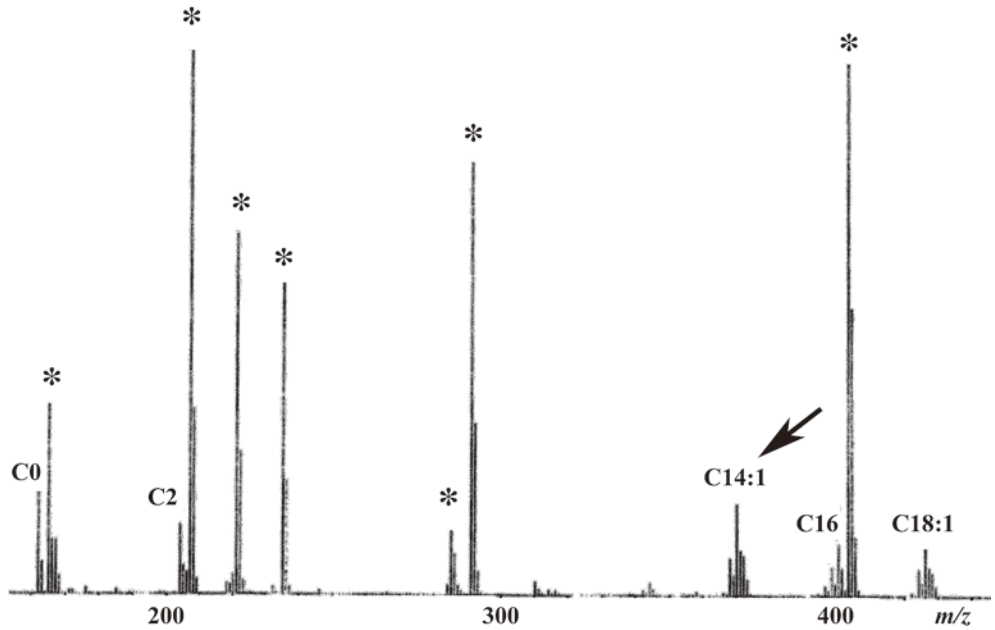

Fig. 1. Serum acylcarnitine profile of the patient obtained by tandem mass spectrometry (MS/MS) analysis. The ion peaks highlighted with an asterisk show the internal standards. Note that elevation of C14:1-AC (C14:1, arrow) indicates VLCAD deficiency.
C0; free carnitine, C2; acetylcarnitine.

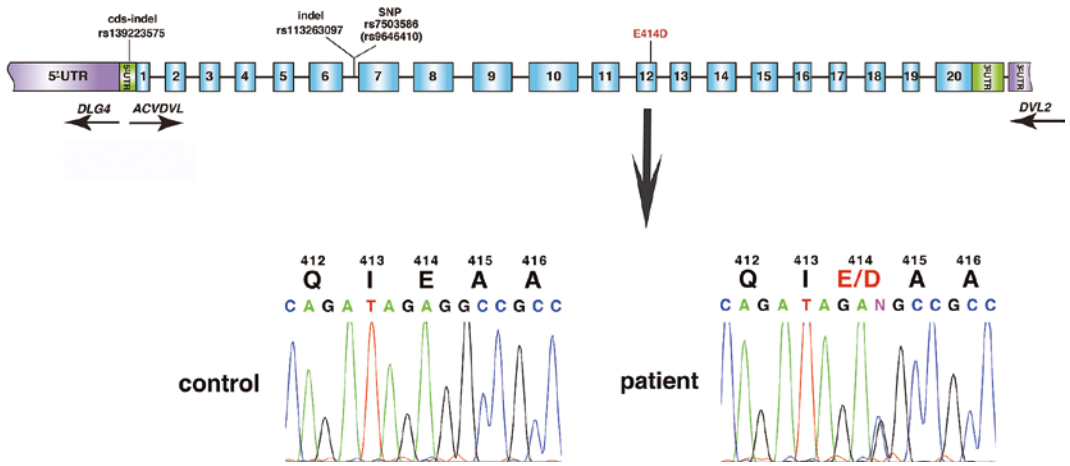

Fig. 2. Genetic analysis of our patient. The *ACADVL* gene that codes VLCAD is located in chromosome 17p13.1 and has 20 exons. We performed direct sequencing from nt 6725713 to 6732487 (NT_010718.16), including whole exons, introns, 5′- and 3′-untranslated region (UTR) of *ACADVL*, a part of 5′-UTR of *DLG4*, and a part of 3′-UTR of *DVL2*. We identified a missense mutation and three nucleotide variations. A heterozygous c.1242G>C (p.E414D) mutation in exon 12 was identified.
rs113263097 is an insert-deletion (indel) variation located in intron 6, which is deletion of one of two 17-nt repeats. rs7503586 (or rs9656410) is a SNP with T>C substitution at the 12th nucleotide of the 17-nt repeat. rs139223575 is located in 5′-UTR, and is a 15-nt insertion.

available build) and the NHLBI Exome Sequencing Project (http://evs.gs.washington.edu/EVS/), we did not find this genomic variation and amino acid substitution. The glutamate residue E414 is located within a helix of the acyl-CoA dehydrogenase C-terminal domain (amino acid positions: 325-473) (Fig. 3). This residue is highly conserved in various species (Fig. 4). To evaluate predicted pathogenicity of the missense mutation E414D, we investigated protein variant effect algorithms using by SIFT, and Polyphen2. These algorithms indicated that p.E414D was predicted to be damaging (SIFT score; 0 and HumDiv score; 0.968). These results suggest that the residue may have an important role in the acyl-CoA dehydrogenase function of VLCAD.

We also identified some genetic variations. There was a single nucleotide polymorphisms (SNP) (Fig. 2): a C>T variation in intron 6 of *ACADVL* (rs7503586 or rs9656410).

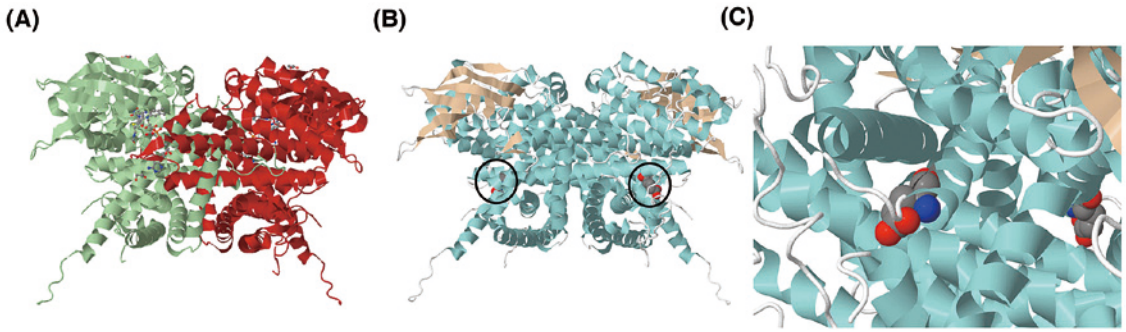

Fig. 3. Three-dimensional structure of the human VLCAD (PDB 2UXW or 3B96). (A) Dimeric structure of the human VLCAD. VLCAD enzyme forms a homodimer (McAndrew et al. 2008). (B) The glutamate residue 414 is located within a helix of the acyl-CoA dehydrogenase C-terminal domain (open circles). Each helix is represented in aqua blue and sheet in apricot. The glutamate residue 414 is shown based on CPK model. Gray, red, and blue spheres represent carbon, oxygen, and nitrogen atom, respectively. (C) Rotated and magnified structure model. All figures were generated using an open source molecule viewer Jmol (http://jmol.sourceforge.net).

	match	AA alignment
Human (*Homo Sapiens*)	414	M D Q G A T D F Q I **E** A A I S K I F G S E
Ape (*Pan Troglodytes*)	414	M D Q G A T D F Q I **E** A A I S K I F G S E
Mouse (*Mus Musculus*)	415	M D Q G F K D F Q I **E** A A I S K I F C S E
Turtle (*Chrysemys Picta*)	337	M D Q G A A D F H T **E** A A M S K I F G S E
Fish (*Danio Rerio*)	419	M D S G A T E F Q I **E** A A I S K I F A S E
Coelacanth (*Latimeria chalumnae*)	271	M D M G A S E F Q I **E** A A I S K I F A S E

Fig. 4. Sequence alignment of a part of acyl-CoA dehydrogenase C-terminal domain of VLCAD in various species. The glutamate residue (human E414) is highly conserved in human, ape, mouse, turtle, fish, and coelacanth (arrow).

This SNP was within another insert-deletion (indel) variation (rs113263097) (Fig. 2). One of two 17-nucleotide repeats was absent. We also found an insertion of the same 15-nucleotide sequence at approximately 50-60 bp upstream of the initiation codon (rs139223575) (Fig. 2). Notably, the nucleotide mutation causing the p.E414D amino acid substitution was heterozygous, whereas all the four genetic variations were homozygous. There were no amino acid deletions and no insertions of intervening sequence (IVS) in the intron-exon boundaries.

Our patient had no weakness and myalgia at discharge. We advised him to avoid exertional exercise and excessive dieting. We prescribed no medications such as L-carnitine, riboflavin, and coenzyme Q10. During subsequent follow-up of over three years, no episodes of rhabdomyolysis occurred, indicating that this case is the mildest form of VLCAD deficiency.

Genomic DNA sample was obtained after receiving informed consent for the clinical diagnosis of VLCAD deficiency and prepared from peripheral blood leukocytes. The Sapporo Medical University Ethics Committee approved this study.

Discussion

VLCAD deficiency was first identified and is the most frequent fatty acid oxidation disorder in Japan (Aoyama et al. 1993; Yamaguchi et al. 1993; Tajima et al. 2008). An adolescent- or adult-onset myopathic form develops isolated skeletal muscle involvements such as rhabdomyolysis and myoglobinuria after exercise or fasting (Andresen et al. 1999). The prognosis of late-onset VLCAD deficiency is relatively good, but recurrent episodes of muscular manifestations persist in 10-20% of the patients (Wilcken 2010).

A diagnosis of VLCAD deficiency is confirmed by elevated C14:1-AC and decreased palmitoyl-CoA dehydrogenase activity. Acylcarnitine analysis by MS/MS analysis has been introduced for newborn mass screening to detect inborn errors of metabolism. The diagnosis of VLCAD deficiency is based on confirmation of decreased palmitoyl-CoA (C16:0) dehydrogenation in tissues. We reported the use of HPLC to detect 2-hexadecenoyl-CoA (C16:1) production from palmitoyl-CoA as dehydrogenase activity of VLCAD (Tajima et al. 2008). Spiekerkoetter et al. (2009) reported that 17 of 20 patients with VLCAD deficiency identified by newborn screening were asymptomatic at the time of diagnosis. These asymptomatic individuals remained symptom-free up to seven years of age. Our patient showed significant reduction of VLCAD activity (approximately 6.3% of control values) (Tajima et al. 2008). On the other hand, previous observations have shown that residual VLCAD activity correlates poorly with clinical severity (Aoyama et al. 1995; Andresen et al. 1996; Vianey-Saban et al. 1998; Pons et al. 2000). This lack of association between biochemical and clinical manifestations could support the hypothesis of multifactorial pathogenesis in VLCAD deficiency. As one explanation for this complex-

ity, abnormal accumulation of long-chain fatty acid (LCFA) metabolites results in increased tissue toxicity to myocardium and skeletal muscle (Corr et al. 1989; Andresen et al. 1996). LCFA intermediates have also been shown to have an inhibitory effect on oxidative phosphorylation and fatty acid oxidation (Kunau et al. 1995; Ventura et al. 1996).

Our patient had a novel missense mutation p.E414D in *ACADVL*. We identified no abnormalities of exon-intron boundary by performing genome sequencing of all exons of *ACADVL*. However, we were not able to investigate the genotypes of the patient's parents. More than 100 mutations such as missense, small deletion, and abnormal splicing due to insertions of IVS have been identified in *ACADVL*, and mutations are distributed all over the gene (Gregersen et al. 2008). Near the mutation site of the present case, two pathogenic substitutions; p.A416T (c.1246G>A) and p.T409M (c.1226C>T), have been reported (Fukao et al. 2001; Isackson et al. 2013). Two single nucleotide variations resulting in an amino-acid change before position 414 have also been identified (rs201606472; c.1237A>G, p.I413V and rs143172658; c.1239A>G, p.I413M). VLCAD is localized in the inner mitochondrial membrane, forming a homodimer. The N-terminal domain of VLCAD (approximately 400 amino acids) shows a high sequence identity and homology with other acyl-CoA dehydrogenases, although approximately 180 amino acid residues in the C-terminal are unique to VLCAD (McAndrew et al. 2008). The C-terminal domain forms a helical bundle that interacts with the N-terminal domain. According to a previous report, Ala416 is directly across from a phenylalanine on helix K, which forms a loop around the catalytic Glu462 (Gobin-Limballe et al. 2010). Certain substitutions could affect catalytic Glu462 by losing the salt bridge.

VLCAD deficiency is inherited in an autosomal recessive manner, with homozygous or compound heterozygous mutations, but some previous reports show cases with a heterozygous missense mutation in *ACADVL* (Andresen et al. 1999; Mathur et al. 1999; Pons et al. 2000; Schymik et al. 2006; Laforet et al. 2009). Even a single missense mutation has been associated with severe clinical phenotypes such as infantile dilated cardiomyopathy and sudden death (Mathur et al. 1999). These findings indicate that VLCAD deficiency due to a missense mutation of *ACADVL* is heterogeneous, and is an insidious disease because of the variable phenotype. We assume that this heterozygous mutation may lead to exhibit transdominant negative effect. Human VLCAD was composed of a homodimer (McAndrew et al. 2008; Gobin-Limballe et al. 2010). A heterozygous mutation may produce an aberrant monomer, resulting in the production of noxious homodimers even one of a pair is normal. Mutated homodimers may be able to induce the disruption of normal molecules and act as dominant negative effect (Veitia 2009).

We speculate that apart from the novel missense mutation, the multiple homozygous nucleotide variations detected in our patient also altered the VLCAD activity and the skeletal muscle vulnerability. Two recent genome-wide association studies of the general population have reported that specific intronic SNPs of short- and medium-chain acyl CoA dehydrogenase (SCAD and MCAD) alter the ratio of acylcarnitine metabolites and insulin release (Gieger et al. 2008; Hornbak et al. 2011). These studies suggest that individuals who have homozygous minor alleles of SCAD or MCAD may have impaired β-oxidation of fatty acids. The ethnic frequency and pathophysiological role of these mutations and variants found in our patient remain unclear, whereas reduced VLCAD activity caused by a heterozygous *ACADVL* mutation may be further impaired directly or indirectly by the interaction between nucleotide variation and environment.

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Conflict of Interest

The authors declare no conflict of interest.

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