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Flavin Adenine Dinucleotide Status and the Effects of High-Dose Riboflavin Treatment in Short-Chain Acyl-CoA Dehydrogenase Deficiency

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ABSTRACT: Short-chain acyl-CoA dehydrogenase deficiency (SCADD) is an inborn error, biochemically characterized by increased plasma butyrylcarnitine (C4-C) concentration and increased ethylmalonic acid (EMA) excretion and caused by rare mutations and/or common gene variants in the SCAD encoding gene. Although its clinical relevance is not clear, SCADD is included in most US newborn screening programs. Riboflavin, the precursor of flavin adenine dinucleotide (FAD, cofactor), might be effective for treating SCADD. We assessed the FAD status and evaluated the effects of riboflavin treatment in a prospective open-label cohort study involving 16 patients with SCADD, subdivided into mutation/mutation (mut/mut), mutation/variant (mut/var), and variant/variant (var/var) genotype groups. Blood FAD levels were normal in all patients before therapy, but significantly lower in the mut/var and var/var groups compared with the mut/mut group. Riboflavin treatment resulted in a decrease in EMA excretion in the mut/var group and in a subjective clinical improvement in four patients from this group. However, this improvement persisted after stopping treatment. These results indicate that high-dose riboflavin treatment may improve the biochemical features of SCADD, at least in patients with a mut/var genotype and low FAD levels. As our study could not demonstrate a clinically relevant effect of riboflavin, general use of riboflavin cannot be recommended. **(*Pediatr Res* 67: 304–308, 2010)**

Short-chain acyl-CoA dehydrogenase (SCAD, EC 1.3.99.2; MIM 606885) deficiency (SCADD; MIM 201470) is an autosomal recessive inborn error of mitochondrial fatty acid oxidation. SCADD is most frequently diagnosed as a result of investigations for developmental delay, epilepsy, behavioral disorders, hypoglycaemia, and hypotonia (1–13), but the diagnosis of SCADD probably has no clinical significance in many individuals (5). SCADD is one of the most common inborn errors of metabolism (5). Although SCADD does not seem to meet newborn screening criteria (5), a committee of the National Academy of Clinical Biochemistry could not reach consensus on recommending against adoption of SCADD in newborn screening programs (14). Therefore, SCADD is still included in newborn screening programs in most US states (15). In addition, potential treatment options for SCADD have never been systematically studied.

SCADD is caused by decreased activity of the first enzyme of the short-chain fatty acid β-oxidation spiral, which catalyzes the dehydrogenation of butyryl-CoA (C4-CoA). When SCAD activity is impaired, C4-CoA will accumulate and is subsequently converted into different metabolites including 1) the corresponding carnitine-ester, *i.e.* butyrylcarnitine (C4-C); 2) the corresponding glycine ester (butyrylglycine); 3) butyrate; and 4) ethylmalonic acid (EMA). C4-C can be measured in blood, whereas EMA can be measured in urine. In general, these two metabolites are both elevated in SCADD, although to different extents.

The diagnosis is confirmed by DNA analysis of the gene that encodes SCAD (*ACADS*) (5). Currently, 38 different mutations have been reported in patients with SCADD (2,3,5,6,10,11,16–18). In addition, two common *ACADS* variants, c.511C>T and c.625G>A have been found in the general population with prevalence of homozygosity of ~0.3 and 5.5%, respectively (19,20). Most patients with SCADD are homozygous or compound heterozygous for one or two of the common *ACADS* variants or for a combination of one of these variants with an *ACADS* mutation (2,3,5,10,11). Both gene variants may play a modifying role in the pathogenesis of clinical SCADD, by conferring susceptibility to clinical disease (10,21).

The SCAD enzyme is a flavoprotein consisting of four subunits, each of which contains one molecule of its cofactor flavin adenine dinucleotide (FAD). Riboflavin, vitamin B2 (7,8-dimethyl-10-ribityl-isoalloxazine), is the precursor of FAD and is predominantly ingested through the consumption of milk and dairy products (22). FAD binding is important not only for the catalytic activity of flavoproteins but also for their folding, assembly, and/or stability (23–25). Because all of the studied *ACADS* mutations and variants result in protein misfolding (3), riboflavin therapy might be particularly efficacious in patients with SCADD if it can stabilize the affected protein. In addition, riboflavin deficiency is a relatively common con-

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Abbreviations: ***ACADS,*** SCAD encoding gene; **C4-C,** butyrylcarnitine; **EMA,** ethylmalonic acid; **FAD,** flavin adenine dinucleotide; **mut/mut,** mutation/mutation; **mut/var,** mutation/variant; **SCADD,** short-chain acyl-CoA dehydrogenase deficiency; **var/var,** variant/variant

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dition (26) and could therefore be a common environmental factor reducing *SCAD* activity in susceptible SCADD individuals, resulting in clinical disease. Profound riboflavin deficiency, typically presenting as angular stomatitis, cheilosis, and glossitis, is common in developing country populations (27,28) but rare in Western societies (29).

Results of riboflavin treatment have previously been reported in only three patients with SCADD (12,13,30). In two of them, riboflavin treatment seemed to be beneficial (12,30). In one of them, clinical improvement persisted after cessation of therapy (12).

The purpose of our study was to systematically assess the FAD status in individuals with SCADD and to evaluate the effects of high-dose riboflavin treatment on the biochemical characteristics and clinical status in a relatively large cohort of patients with SCADD and to compare effects between the different *ACADS* genotypes.

SUBJECTS AND METHODS

Study design and patients. We conducted a prospective open-label cohort study between January 2003 and January 2008. Sixteen patients with SCADD, all initially investigated because of clinical symptoms and diagnosed with SCADD after sequence analysis of all exons and flanking intronic sequences of the *ACADS* gene had been performed (Tables 1 and 2), were included in the study. All patients but patients 11 and 16 were part of the Dutch SCADD cohort that has been previously described (5). Patients were classified into three different genotype groups: 1) *ACADS* mutations on both alleles [mutation/mutation (mut/mut) group; $n = 3$]; 2) an *ACADS* mutation on one allele and an *ACADS* variant on the other allele or both alleles [mutation/variant (mut/var) group; $n = 8$]; and 3) *ACADS* variants on both alleles [variant/variant (var/var) group; $n = 5$] (Table 1).

This study was approved by the Institutional Review Board of the Academic Medical Center and informed consent was obtained from all parents.

Treatment and assessment. Riboflavin was administered as 10 mg/kg body weight per day, divided into three doses with a maximum of 150 mg/d, and ingested during meals. Before (d 0) and 5 wk after starting treatment (d 35), blood was obtained for C4-C and FAD analyses, and urine was obtained for determination of FAD excretion. During the 7 d preceding d 0 and d 35, five early morning voids were collected to determine EMA levels in urine. All urine and blood samples were obtained after an overnight fast and, for samples collected during riboflavin therapy, before the morning riboflavin dose.

Blood and urine analyses. The FAD status was determined by analysis of whole blood and urine using HPLC (31). Acylcarnitine profiles were determined using electrospray tandem mass spectrometry (32). Urine samples were stored at −20°C until analysis and were analyzed for organic acids by gas chromatography/mass spectrometry (33).

Clinical signs and symptoms. Both before and 5 wk after the start of riboflavin therapy, a medical history was taken. At the 5 wk appointment, parents were asked if they had noticed any changes in the condition of their child during the period of riboflavin treatment.

Statistical Analysis. The Kruskall-Wallis test was used to compare the three different genotype groups and the Mann-Whitney test to compare two different groups. The Wilcoxon signed rank test was used to compare baseline values with values obtained during treatment. The level of significance was set at $p < 0.05$. Analyses were performed using Graphpad Prism 3.0.

RESULTS

FAD status and FAD response to riboflavin treatment. Blood FAD concentrations before riboflavin treatment were within the normal reference range in all but one patient (patient 2, who received high caloric tube feeding, Table 1). A significant difference in blood FAD concentrations was observed among the three genotype groups before the start of treatment, with the lowest FAD concentrations observed in the mut/var and var/var groups (Table 1). During treatment, median blood and urine FAD concentrations in all the patients with SCADD increased significantly and did not differ between the three genotype groups (Table 1).

EMA and C4-C in response to riboflavin treatment. Median EMA excretion decreased significantly only in the mut/var group (Table 1). None of the genotype groups showed a significant decrease in blood C4-C concentration (Table 1). However, in five of the eight patients in the mut/var group a biochemical response, defined as a clear decrease in plasma C4-C levels, in response to riboflavin was observed (Table 1). In four of them, this C4-C decrease was accompanied by a distinct decrease in EMA excretion. Of note, EMA levels in the fifth patient were only slightly increased before treatment (Table 1). In three of these five biochemically responding patients, blood FAD levels were measured before and during riboflavin treatment. The increase in FAD levels was significantly higher compared with the increase in plasma FAD levels in the patients with SCADD who did not respond to riboflavin (Table 1).

Clinical signs and symptoms. During treatment, all patients (and/or their parents) reported a change in color of the urine. In four of the patients (patients 4, 7, 8, and 10), all responding biochemically to riboflavin, a slight clinical improvement was reported 5 wk after initiation of riboflavin treatment (Table 2). The improved clinical condition persisted in all four patients after discontinuation of treatment and during a follow-up of 2 y.

DISCUSSION

Our study is the first to systematically examine the efficacy of a potential treatment for SCADD, one of the more common inborn errors of metabolism. SCADD is included in newborn screening programs in most of the US states, although it has questionable clinical relevance and no treatment has been evaluated.

A high dose of riboflavin (10 $mg \cdot kg^{-} \cdot d^{-}$, with a maximum of 150 mg/d) was administered to patients in this study. This dose was thought to be sufficient to obtain the maximal attainable FAD levels in these patients, as there is little additional absorption of riboflavin for single doses greater than 30 mg (34). The significant increase during treatment in blood FAD concentrations and urinary FAD excretion in the total study group supports this (Table 1).

None of the patients had decreased blood FAD concentrations at baseline. However, the median blood FAD concentrations in the mut/var and var/var groups were significantly lower compared with the concentrations in the mut/mut group. Furthermore, the patients who responded biochemically to riboflavin were among the patients with the lowest blood FAD concentrations. Finally, these patients had a significantly higher increase in blood FAD compared with the biochemically nonresponding patients. Our results suggest that a relatively low FAD status may be involved in the expression of biochemical features of SCADD. This leads us to hypothesize that individuals with SCADD with a mut/mut genotype will be identified by biochemical screening of urine and/or plasma irrespective of their FAD concentrations,

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Table 1. *Genotype, FAD levels in blood and urine, median EMA levels in urine, and C4-C levels in plasma at baseline and after 5 wk of riboflavin treatment in 16 Dutch patients with SCADD*

Patient				Blood FAD (μmol/L)*			Urine FAD (mg/L)			Median (range) EMA (μmol/mmol creatinine) in urine†			C4-C (μmol/L) in plasma‡		
		Mutation analysis		Day			Day			Day			Day		
No.	Sex/age (y)	Allele 1	Allele 2	0	35	*p*	0	35	*p*	0	35	*p*	0	35	*p*
Mutation/mutation group															
1	M/4	c.1138C>T	c.1138C>T	0.34	0.31		2.75	NA		96 (52–116)	96 (70–115)		5.20	4.21	
2§	M/2	c.988C>T, c.625G>A	c.1147C>T	0.41	0.37		7	8.8		159.5 (158–161)	150.5 (140–177)		5.22	5.85	
3	F/7	c.988C>T, c.625G>A	c.1147C>T	0.34	0.35		1.7	6.7		67.5 (57–78)	72 (60–75)		4.52	5.10	
Median values				0.34‖	0.35¶		2.75¶	7.75¶		96	84	NS	5.2	5.1	NS
Mutation/variant group															
4	**M/12**	**C136C>T, c.625G>A**	**c.625G>A**	**0.22**	**0.35****		**35**	**34**		**413 (352–448)**	**48 (43–51)**		**5.66**	**0.59**	
5	F/4	c.1058C>T	c.625G>A	0.27	0.30		1.39	7.00		28 (19–45)	30 22–40)		1.00	2.40	
6	**M/3**	**c.1058C>T**	**c.625G>A**	**NA**	**0.34**		**NA**	**31.00**		**30 (28–38)**	**24 (19–25)**		**1.76**	**0.91**	
7	**F/6**	**c.1058C>T**	**c.625G>A**	**0.28**	**0.35****		**0.32**	**14**		**39 (26–42)**	**15 (11–24)**		**0.71**	**0.48**	
8	**M/1**	**c.1058C>T**	**c.625G>A**	**NA**	**NA**		**NA**	**NA**		**74 (60–110)**	**57 (42–59)**		**1.73**	**0.80**	
9	F/3	c.1058C>T	c.625G>A	0.27	0.26		1.00	5.30		23 (19–39)	19 (19–42)		1.38	1.66	
10	**F/14**	**c.IVS1–6C>A**	**c.625G>A**	**0.22**	**0.29****		**0.80**	**9.50**		**10 (8–16)**	**11 (8–15)**		**0.96**	**0.47**	
11	M/7	c.449C>T	c.625G>A	0.29	0.32		0.52	1.6		20 (11–32)	16 (13–20)		0.66	0.75	
Median values				0.27‖	0.32¶		0.9¶	9.5¶		29	21.5	0.039	1.19	0.78	NS
Variant/variant group															
12	M/2	c.625G>A	c.625G>A	0.28	0.30		0.79	25.33		10 (9–10)	18 (11–35)		0.52	0.46	
13	M/3	c.625G>A	c.625G>A	0.33	0.31		2.30	6.81		17 (5–28)	13 (11–21)		0.64	0.53	
14	F/4	c.625G>A	c.625G>A	0.22	0.29		1.92	3.80		7 (5–13)	7 (6–12)		0.45	0.61	
15	M/17	c.625G>A	c.625G>A	0.28	0.37		0.29	NA		7 ((5–7)	5 (4–7)		0.36	0.36	
16	F/1	c.625G>A	c.625G>A	0.26	0.31		0.39	4.5		9 (9)	8 (8–10)		0.40	0.52	
Median values				0.28‖	0.31¶		0.75¶	5.66¶		9	8	NS	0.45	0.52	NS
Median values of the entire cohort				0.28	0.31	0.035	1.2	7	0.001						

Data of riboflavin-responsive patients are in bold type.

* Normal range of blood FAD: 0.20–0.36 μmol/L.

† Normal range of EMA: 0–8 μmol/mmol creatinine, median and range of EMA calculated from five early morning voids collected within the preceding 7 d.

‡ Maximum normal concentration of C4-C: 0.58 μmol/L.

§ Subjected to high-calorie tube feeding.

‖ Significant difference between the 3 genotype groups ($p = 0.03$).

¶ No significant difference between the 3 genotype groups.

** Significantly higher increase in FAD levels as compared with the other, nonresponding, patients with SCADD (0.07 μmol/L and 0.02 μmol/L, respectively, $p = 0.04$).

NA, not analyzed; NS, not significant.

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Table 2. *Baseline clinical phenotype and changes in the clinical conditions of 16 Dutch SCADD patients after 5 wk of riboflavin treatment*

Patient			
No.	Sex/Age (y)	Clinical phenotype	Change in clinical condition during treatment as reported by parents
Mutation/mutation group			
1	M/4	Currently none (transient infantile hepatic dysfunction)	None reported
2	M/2	Developmental delay, epilepsy, food refusal, hypertonia	None reported
3	F/7	Currently none (transient feeding problems in infancy)	None reported
Mutation/variant group			
4	**M/12**	**Epilepsy, mild developmental delay**	**More active**
5	F/4	Epilepsy, mild developmental delay	None reported
6	**M/3**	**Mild developmental delay, hypoglycemia**	**None reported**
7	**F/6**	**Mild developmental delay, exercise intolerance**	**Slight decrease of exercise intolerance**
8	**M/1**	**Epilepsy, severe developmental delay, dysmorphism, hypotonia**	**Slight decrease of hypotonia, increase in development**
9	F/3	Hypoglycemia	None reported
10	**F/14**	**Fatigue**	**Disappearance of fatigue**
11	M/7	Developmental delay, behavioral disorders, feeding problems	None reported
Variant/variant group			
12	M/2	Hypoglycemia	None reported
13	M/3	Epilepsy	None reported
14	F/4	Mild developmental delay	None reported
15	M/17	Developmental delay, dysmorphic features, scoliosis	None reported
16	F/1	Failure to thrive	None reported

Data of riboflavin-responsive patients (see Table 1) are in bold type.

whereas individuals with SCADD with either a mut/var or a var/var genotype may only be identified by metabolic screening if their FAD levels are low, albeit still in the normal range. In these latter groups, relatively low FAD levels may result in a further decrease of SCAD activity resulting in the characteristic biochemical signs of SCADD. This hypothesis is supported by our observation that patients 4, 7, and 10 (all with a mut/var genotype) achieved nearly normal C4-C concentrations during riboflavin treatment. These findings suggest that patients with these SCADD genotypes may not have been identified by screens for increased C4-C concentrations, the method applied for newborn screening for SCADD, when their FAD levels were high. The number of patients in our study was too small to fully test this hypothesis. It might be of interest to assess the FAD status of individuals with SCADD identified by newborn screening in future studies.

Four of the five patients who responded biochemically to riboflavin showed a slight clinical improvement as reported by their parents. As their initial FAD status was within the normal range and because they had no signs or symptoms specific for riboflavin deficiency, the reported clinical response cannot be explained by the correction of true riboflavin deficiency. The observed clinical improvement may, however, be explained by a placebo effect. This is supported by the observation that none of these patients reported any deterioration in their clinical condition after cessation of riboflavin therapy during a follow-up period of 2 y.

None of the patients who were homozygous for the *ACADS* c.625G>A variant and without an additional *ACADS* mutation demonstrated a biochemical improvement while on riboflavin treatment. However, the biochemical abnormalities characteristic for SCADD were only mild at baseline in most of these patients. All patients responding to riboflavin had a mut/var genotype. Our study failed to reveal a correlation between a specific *ACADS* mutation and responsiveness to riboflavin treatment (Table 1). This implies that the mut/var genotype may be related to functional SCADD based on decreased FAD affinity or SCAD protein instability associated with the presence of the c.625G>A variant. A low FAD status in combination with the c.625G>A variant may thus be the determinant factors for riboflavin responsiveness.

Although we demonstrated that high-dose riboflavin treatment leads to biochemical improvement in a subgroup of patients with SCADD, it is not clear that it leads to any improvement in clinical disease. This can only be addressed by a properly conducted and preferentially blinded trial of riboflavin in patients with a mut/var genotype, focusing on the clinical efficacy of such an intervention. Such a treatment could be especially effective in those patients who have a relatively low FAD status at baseline. Riboflavin cannot be recommended as a general treatment in SCADD.

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