Short-chain acyl-CoA dehydrogenase deficiency
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Curriculum Vitae

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In 1995, she started her training in pediatrics at the Vrije Universiteit Amsterdam (Prof.dr. A.J.P. Veerman and Prof.dr. J.J. Roord), of which she worked 16 months in the Deventer Ziekenhuizen (the late dr. H. Hol). In 2002, she started working as a pediatrician in the Gelre Ziekenhuizen Apeldoorn.

In 2005 onward she was registered as a pediatrician metabolic diseases. Meanwhile she had started the studies on short-chain acyl-CoA dehydrogenase deficiency which resulted in this thesis. In May 2008, she started working as a medical researcher, performing educational work. From October 2009 onward, she works as a pediatrician in the Gelre Ziekenhuizen Apeldoorn.

Bianca van Maldegem is married to Peter Zwaan and they have three children: Linde (1998), Jard (2000), and Marke (2002).
Short-chain acyl-CoA dehydrogenase deficiency

Bianca T. van Maldegem
Short-chain acyl-CoA dehydrogenase deficiency

ACADEMISCH PROEFSCHRIFT

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op gezag van de Rector Magnificus prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties ingestelde commissie, in het openbaar te verdedigen in de Aula der Universiteit op vrijdag 28 januari 2011, te 11:00 uur

door

Bianca Tanja van Maldegem

geboren te Arnhem
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                 Prof. dr. R.J.A. Wanders  

Co-promotor:    Dr. H.R. Waterham  

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Faculteit der Geneeskunde
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Chapter 1

Introduction
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3 Outline of the thesis
1 Short-chain acyl-CoA dehydrogenase and its role in energy metabolism

Short-chain acyl-CoA dehydrogenase (SCAD; MIM 606885) is one of many enzymes involved in energy metabolism in humans. SCAD participates in the oxidation of fatty acids in mitochondria, a pathway central to the provision of energy for the mammalian organism. Fatty acid oxidation (FAO) occurs at the level of the β-carbon and is therefore also referred to as fatty acid β-oxidation. FAO is of great importance for the supply of energy during fasting, when it provides up to 80% of the total energy requirements, and during moderately intense exercise. In addition, the heart prefers fatty acids and FAO-derived ketone bodies as substrates for the production of energy. Fatty acids, the substrate for FAO, are stored in adipose tissue as triacylglycerols and are released during lipolysis (Figure). This is initiated by the activation of hormone-sensitive lipase. Under fasting conditions, lipase is activated by a decrease in the insulin:glucagon ratio, whereas during exercise an increase in epinephrine stimulates lipase activity. The process of lipolysis involves hydrolysis of triacylglycerols into glycerol and free fatty acids (FFA). Because the majority of the stored fatty acids in triacylglycerols are long-chain fatty acids containing 16-20 carbon atoms, a majority of the released FFA are consequently long-chain fatty acids. After release into the circulation, these long-chain fatty acids are taken up by various tissues for their energy supply.

The actual β-oxidation process occurs within the mitochondria, which requires the FFA to first be transported across the mitochondrial membrane. The first step needed for the initiation of FAO is the activation of long-chain FFA to their long-chain acyl-CoA esters in the cytoplasm (Figure). Because long-chain acyl-CoA esters cannot cross the mitochondrial inner membrane, long-chain acyl-CoA’s first need to be converted into the corresponding acylcarnitine esters by the enzyme carnitine palmitoyl-transferase I. The mitochondrial carnitine acylcarnitine translocase mediates the transfer of the acylcarnitines into the mitochondrion, followed by carnitine palmitoyl-transferase II which regenerates the long-chain acyl-CoA’s, thereby releasing free carnitine. Carnitine is transported back into the cytosol by the acylcarnitine translocase, thus completing the carnitine shuttle (Figure). Medium and short-chain fatty acids can enter the mitochondria without the support of the carnitine cycle.

Once inside the mitochondrion the acyl-CoA’s can be degraded by mitochondrial β-oxidation. This involves the process of chain shortening by repetitive β-oxidation cycles. Via a series of 4 consecutive enzymatic reactions an acyl-CoA is shortened by 2 carbons at the end of one full β-oxidation cycle, which results in the release of an acetyl-CoA unit (Figure). The enzymes involved in these 4 steps, each group of which is chain-length specific, are acyl-CoA dehydrogenases, 2-enoyl-CoA hydratases, L-3-hydroxy-acyl-CoA dehydrogenases and 3-oxoacyl-CoA thiolases. The SCAD enzyme is one of the acyl-CoA dehydrogenases and catalyzes the first step of a specific part of the β-oxidation cycle:
Figure. Metabolism of long-chain fatty acids in liver in short-chain acyl-CoA dehydrogenase deficiency. Lipolysis is initiated by the activation of hormone-sensitive lipase, which is activated by a decrease in the insulin:glucagon ratio during fasting and an increase in epinephrine during exercise. The process of lipolysis involves hydrolysis of triacylglycerols into glycerol and long-chain fatty acids, such as palmitate (16 carbons). After release into the circulation, these long-chain fatty acids are taken up by the liver. They are activated to their CoA esters in the cytoplasm of liver cells and subsequently converted into the corresponding acylcarnitine esters by the enzyme carnitine palmitoyl-transferase I (CPT-1). The mitochondrial carnitine acylcarnitine translocase (CACT) mediates the transfer of the acylcarnitines into the mitochondrion, where carnitine palmitoyl-transferase II (CPT-2) regenerates the acyl-CoA’s, thereby releasing free carnitine. Carnitine is transported back into the cytosol by the acylcarnitine translocase, thus completing the carnitine shuttle. Inside the mitochondrion the fatty acid is degraded during the process of chain shortening by repetitive β-oxidation cycles. Via a series of 4 consecutive enzymatic reactions an acyl-CoA is shortened by 2 carbons at the end of one full β-oxidation cycle, which results in the release of an acetyl-CoA unit and the formation of one molecule of flavin adenine dinucleotide (FAD)H2 and one molecule of nicotinamide adenine dinucleotide (NAD)H. These reduced nucleotides are used directly for the synthesis of adenosine triphosphate (ATP) by oxidative phosphorylation. The acetyl-CoA units will predominantly be used for the formation of ketone bodies (KB). The KB can be utilized for generation of energy by other tissues, as KB can be reconverted into acetyl-CoA units.

Starting with a long chain fatty acyl-CoA with 16 carbons, it will take 6 β-oxidation cycles to produce a short-chain (4 carbons) acyl-CoA. The final β-oxidation cycle is initiated by short-chain acyl-CoA dehydrogenase (SCAD), and normally results in the production of another 2 molecules of acetyl-CoA. In the case of SCAD deficiency, this final β-oxidation cycle cannot occur, or only partially. Consequently, ATP production from reduced nucleotides will be diminished with maximally 5/35 part (14 %) and KB formation from acetyl-CoA with maximally 2/8 part (25%). At the same time, the substrate for SCAD, butyryl-CoA (C4-CoA) will accumulate and be converted via alternative pathways into different metabolites including butyrylcarnitine (C4-C), butyrate, butyrylglycine, and ethylmalonic acid (EMA).
the final degradation of short-chain acyl-CoA's into acetyl-CoA units. During this first dehydrogenation step, enzyme-bound flavin adenine dinucleotide (FAD) is reduced into FADH$_2$ (Figure), after which the reducing equivalents are fed into the respiratory chain at the level of ubiquinone via electron transfer flavoprotein (ETF) and ETF-dehydrogenase. At the second dehydrogenation step, nicotinamide adenine dinucleotide (NAD$^+$) is reduced into NADH (Figure), which then enters the respiratory chain at the level of complex I. In this way, the generated reducing equivalents are funneled into the respiratory chain, which ultimately leads to the production of adenosine triphosphate (ATP).

Starting with a long chain fatty acyl-CoA with 16 carbons, it takes 6 β-oxidation cycles to produce a short-chain (4 carbons) fatty acyl-CoA (Figure). The final β-oxidation cycle, of which SCAD catalyzes the first enzymatic reaction, results in the production of another 2 molecules of acetyl-CoA. The acetyl-CoA molecules produced during the whole β-oxidation process can be used by the tricarboxylic cycle and respiratory chain for the direct production of ATP in cells of different tissues. However, some tissues, in particular the liver and to a lesser extent the kidneys and small intestine, can utilize the acetyl-CoA produced by β-oxidation for the formation of ketone bodies (KB).$^1$ These KB can be utilized for the generation of energy by other tissues, because KB can be reconverted into acetyl-CoA units. Under fasting conditions, KB can be used as an alternative substrate for energy metabolism, particularly by the brain.

2 Short-chain acyl-CoA dehydrogenase deficiency

2.1 Metabolic and potential pathophysiological consequences

In the case of SCAD deficiency (SCADD; MIM 201470), the oxidation rate of the short-chain fatty acyl-CoA, butyryl-CoA (C4-CoA) is decreased. This implies a reduction in the amount of reducing equivalents and acetyl-CoA produced during the final β-oxidation cycle. Consequently, this will result in lower production of ATP and KB in liver during fasting (Figure). Because the preceding β-oxidation cycles will produce 86% and 75% of the maximal achievable ATP and KB respectively, the total production of ATP and KB could be reduced by up to 14% and 25% respectively, depending on the residual SCAD activity. Theoretically, this may have several consequences. First, the resulting lower production of KB may reduce the glucose sparing capacity. Furthermore, gluconeogenesis might be attenuated due to lowered availability of reducing equivalents.$^2$ These mechanisms may induce hypoglycemia by the same key mechanisms hypothesized for long- and medium-chain FAO disorders. Furthermore, the production of ATP may be reduced in tissues that depend on FAO. Because FAO is especially important for the generation of ATP in heart and skeletal muscle, a chronic inability to sustain sufficiently high ATP levels might induce muscle damage similar to the muscle damage observed in long-chain FAO disorders. At the same time, the substrate for SCAD, C4-CoA, will accumulate and subsequently be
converted via alternative pathways into different metabolites: butyrylcarnitine (C4-C), butyrate, butyrylglycine, and ethylmalonic acid (EMA) (Figure). All of these metabolites may have toxic effects, which could result in the development of clinical symptoms. Indeed, this has been suggested for EMA and butyrate.\textsuperscript{3,4}

2.2 Molecular biology

The SCAD-encoding gene, \textit{ACADS}, has been localized to the terminal region of the long arm of chromosome 12, spans approximately 13 kb and consists of 10 exons.\textsuperscript{5} The SCAD enzyme is a flavoprotein consisting of 4 identical subunits, each of which contains one molecule of its co-factor, FAD. As of 2006, 17 different mutations had been reported in patients with SCADD.\textsuperscript{6-11} Two of these mutations, c.625G>A and c.511C>T, are remarkably common and generally referred to as gene variants.\textsuperscript{7,8,12,13} These gene variants are considered to confer susceptibility for clinical disease,\textsuperscript{7} and have been found in the US population with a remarkably high prevalence. Nagan et al. reported homozygosity and heterozygosity of approximately 0.3% and 5.6%, respectively, for the c.511C>T variant and 5.6% and 31.8% for the c.625G>A variant.\textsuperscript{12} Corydon et al. postulated that certain genetic, cellular, and environmental factors are involved in reducing the catalytic activity of these variant enzymes below a critical threshold leading to the onset of clinical symptoms.\textsuperscript{7}

2.3 Diagnostic tests and definition

Increased concentrations in body fluids of the metabolites derived from C4-CoA, which is the substrate for SCAD and accumulates in SCADD, suggest the presence of SCADD. In particular C4-C, measured in blood and EMA, measured in urine, are used as biochemical markers for SCADD.

The diagnosis of SCADD may be confirmed by enzyme activity measurements in muscle, fibroblasts, and/or lymphocytes, and by DNA studies.\textsuperscript{7,10,14-19} Enzyme analysis in fibroblasts and lymphocytes, however, have demonstrated remarkably high residual SCAD activity in several SCADD individuals.\textsuperscript{10,17,20} Furthermore SCAD activity measurements in fibroblasts and lymphocytes may give inconsistent results.\textsuperscript{7,8} Although the enzyme assay might be more reliable when performed in muscle biopsy specimens,\textsuperscript{17} a muscle biopsy is generally considered too invasive for routine testing for SCADD. Therefore, DNA analysis is considered to be the preferable method for confirming the diagnosis of SCADD.

For our studies, we decided to classify patients as having SCADD only when mutations and/or variants were present on both \textit{ACADS} alleles in combination with increased C4-C and/or EMA under non-stressed conditions on at least 2 occasions.

2.4 Clinical symptoms and prevalence

From 1987, when the very first patient with SCADD was described by Amendt and colleagues,\textsuperscript{14} to 2006, 26 patients were reported in literature. The 4 patients in whom the diagnosis of SCADD was not genetically confirmed are excluded from this review.
This leaves 22 genetically confirmed cases that were reported in the literature during this period.\textsuperscript{4;6-10;14-18;20-22}

The first reported SCADD patient was subsequently genetically characterized by Naito et al. in 1990.\textsuperscript{6} This female patient was reported to suffer from lethargy, hypertonia and circulatory problems with metabolic acidosis in the first week of life. Although she was reported to show normal growth and development without recurrence of metabolic acidosis up to the age of 2 years, Bhala et al. reported her death in 1995, without giving any further clinical details.\textsuperscript{20} As this publication included another SCADD patient who died after initial presentation with severe skeletal hypotonia, a picture of a devastating disorder was suggested. Remarkably however, Bhala’s publication contained another 2 cases, that initially presented with “possible hyperactivity” and “probable seizure activity”, but in which follow-up was reported to be normal.

Subsequently a large study by Corydon et al.,\textsuperscript{7} and several case reports were published demonstrating that SCADD is predominantly associated with developmental delay, hypotonia, epilepsy, and hypoglycemia as well as, in isolated cases, with dysmorphic features, vomiting, hepatic dysfunction after premature delivery, and bilateral optic atrophy.\textsuperscript{4;8-10;15-17;20-22} In addition, one case report suggested an association between SCADD and acute fatty liver of pregnancy in the mother.\textsuperscript{18} Again a striking difference was observed in the outcome of the SCADD patients: 6 of the 8 patients in whom outcome was documented, were reported to have a normal outcome and 1 out of 8 died.\textsuperscript{4;9;10;17;18;21;22}

SCADD originally appeared to be a rare disorder because in the initial 19 years after the first report, only 22 genetically confirmed cases were reported in the literature. However, the potential role of the common gene variants in the development of clinical SCADD suggested a higher prevalence of the disorder.

2.5 Newborn screening

With the development of electrospray tandem mass spectrometry, many countries worldwide have expanded their newborn screening programs or are preparing to do so in the near future. In the Netherlands, SCADD is not included in the newborn screening program, but screening for SCADD had become part of newborn screening programs in 35 of 51 states in the U.S. and in most Australian states.\textsuperscript{23;24} Doubts about the indication for screening for SCADD have been expressed by the Newborn Screening Expert Group of the American College of Medical Genetics, acknowledging the lack of evidence related to the availability of a treatment and a poorly understood natural history.\textsuperscript{25} With the evolution of newborn screening, 3 policy documents have influenced newborn screening programs.\textsuperscript{26} The latest one, the selection criteria for screening by Frankenburg,\textsuperscript{27} was published in 1974 and states 8 criteria based on the report by Wilson and Junger.\textsuperscript{28} The first 3 criteria state that 1) the disease or condition screened for should be serious or potentially serious, 2) the condition should be relatively common and 3) it
must be possible to differentiate diseased from non-diseased individuals.\textsuperscript{27} It remains to be established if SCADD fulfils these criteria.

2.6 Therapeutic options

Apart from a few isolated case reports, the efficacy of a potential treatment has never been systematically studied in a group of SCADD patients. Some case reports described the efficacy of riboflavin therapy.\textsuperscript{15,16,22} Riboflavin is the precursor of FAD, the co-factor for SCAD. In 2 of the 3 SCADD patients described, riboflavin therapy appeared to be beneficial.\textsuperscript{15,22} However, in one of them, clinical recovery continued after cessation of therapy.\textsuperscript{15}

In addition, it is generally believed that, like in other FAO disorders, patients should be recommended to avoid fasting. However, no studies have been undertaken to support this assumption.

3 Outline of the thesis

As described in the previous sections, SCADD is a relatively newly recognized inborn error of metabolism for which many issues still need to be elucidated. First of all, the disorder was initially assumed to be rare, but the commonly found \textit{ACADS} variants suggest that SCADD might be far more prevalent. Secondly, the spectrum of clinical symptoms in previously reported patients is remarkably broad, which necessitates further exploration of the clinical phenotypes. In addition, potential correlations between molecular and biochemical results on the one hand and clinical features on the other hand need to be explored. Thirdly, as the pathophysiology of SCADD is still speculative, more knowledge on pathophysiological mechanisms of this puzzling disorder should be obtained. Finally, well-designed studies on the potential therapeutic efficacy of riboflavin are needed.

We decided to study the clinical, biochemical, genetic, epidemiological, pathophysiological, and therapeutic aspects of this inborn error of metabolism in order to increase knowledge of this intriguing disorder and to provide better care for the patients.

In order to determine the prevalence of the c.625G>A \textit{ACADS} variant in the Netherlands, we analyzed 1036 newborn screening cards (chapter 2). To extend our knowledge of the clinical, biochemical, and genetic features, we studied a group of 31 Dutch SCADD patients and their SCADD relatives (chapter 3). In addition we estimated the prevalence of SCADD and discussed the indication for the inclusion of SCADD in newborn screening programs (chapter 3). To gain insight into pathophysiological consequences and to assess the indication for fasting tests, we retrospectively studied the results of 15 fasting and 6 fat-loading tests in 15 SCADD patients (chapter 4). Searching for an additional factor associated with the development of SCADD and aiming for a potential therapy, we assessed the FAD status and evaluated the effects of riboflavin supplementation.
in a prospective open-label cohort study involving 16 SCADD patients (chapter 5). In addition we evaluated the biochemical response to exercise in 3 SCADD patients and assessed whether high-dose riboflavin therapy exerts any effects on the observed response in one of them (chapter 6). In a review on the clinical aspects of SCADD, we summarized and discussed the relevant literature (chapter 7). In view of the debate on the clinical significance of SCADD and to further estimate the extent of the Dutch SCADD population, we studied the prevalence of the 2 common ACADS variants and the most common ACADS mutation in the Netherlands, as well as the relationship between these variants and mutation and/or increased C4-C and epilepsy, one of the most frequently reported symptoms in SCADD (chapter 8). Finally, we discussed the results of the studies stated above, provided an overview of newly identified and previously reported ACADS mutations, and presented a diagnostic guideline on increased EMA and C4-C (chapter 9).
References

8. Gregersen N, Winter VS, Corydon MJ et al. Identification of four new mutations in the short-chain acyl-CoA dehydrogenase (SCAD) gene in two patients: one of the variant alleles, 511C-->T, is present at an unexpectedly high frequency in the general population, as was the case for 625G-->A, together conferring susceptibility to ethylmalonic aciduria. Hum Mol Genet. 1998;7:619-627.
Chapter 2

The 625G>A SCAD gene variant is common but not associated with increased C4-carnitine in newborn blood spots

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Abstract

The c.625G>A variant of the short-chain acyl-CoA dehydrogenase (SCAD) gene is considered to confer susceptibility for developing “clinical SCAD deficiency (SCADD)” and appears to be common in the general population. To determine the frequency of the c.625G>A variant in the Netherlands, we analyzed 1036 screening cards of 5- to 8-day-old newborns and found 5.5% homozygous and 31.3% heterozygous for the c.625G>A variant. An increased blood/plasma C4-carnitine (C4-C) concentration is considered to be one of the biochemical characteristics of SCADD. To explore the correlation of C4-C levels with the c.625G>A variant, we determined the C4-C concentration, as well as the ratio of C4- to free carnitine, in blood spots from newborns, who were detected as homozygous, heterozygous, or non-carriers for the gene variant. No significant differences were found between these groups.

Our study demonstrates a high frequency of the c.625G>A SCAD gene variant in the Dutch population, but no correlation to significantly increased C4-C levels in blood spots taken between the 5th and 8th days of life. This latter observation might be the result of the relatively late timing of neonatal screening in our country, implying fatty acid oxidation disorders may be missed at that stage. If the c.625G>A variant is associated with clinical SCADD, the high frequency of the variant suggests a possible involvement of SCADD in the pathogenesis of common disorders, probably in relation to other genetic and/or environmental factors. However, homozygosity for the c.625G>A variant might be only a biochemical phenomenon, representing a non-disease.
Introduction

Short-chain acyl-CoA dehydrogenase (SCAD; MIM 606885) deficiency (SCADD, MIM 201470) is an autosomal recessive inborn error of mitochondrial fatty acid β-oxidation. SCAD is the first enzyme of the short-chain β-oxidation spiral, catalyzing the dehydrogenation of C4 and C6 fatty acids. When its activity is impaired, its substrate butyryl-CoA (C4-CoA) will accumulate. This C4-CoA can be converted into the corresponding carnitine-ester (C4-Carnitine (C4-C)) and into ethylmalonic acid (EMA). The latter conversion proceeds via two steps, catalyzed by propionyl-CoA carboxylase and acyl-CoA hydrolase. The accumulating C4-CoA thus results in increased levels of C4-C and EMA, that can be measured in blood and urine respectively.

Approximately 20 patients with enzymatically proven SCADD have been reported in the literature and clinical signs and symptoms appear to be variable or, in one family, even absent. Hypotonia, developmental delay, epilepsy, and ketotic hypoglycemia are the most frequently reported signs and symptoms.

The SCAD-encoding gene (ACADS) has been localized in the terminal region of the long arm of chromosome 12, spans approximately 13 kb, and consists of 10 exons. Twelve different mutations have been found in relation to SCADD. In addition, a high frequency of two different variant alleles, the c.511C>T and, even more frequent, the c.625G>A variant, has been found. The c.625G>A ACADS variant is considered to confer susceptibility for developing “clinical SCADD”. Because the c.625G>A variant has been demonstrated to occur with a high frequency in different populations, one may question its causative relation to clinical SCADD, which still is considered to be a relatively rare inborn error of metabolism.

To determine the prevalence of the c.625G>A ACADS variant in the Netherlands, we analyzed newborn screening cards for this variant allele by a restriction fragment length polymorphism (RFLP) assay. We found 5.5 % to be homozygous and 31.3 % heterozygous for this ACADS variant. In addition, we explored whether there is a correlation between this supposed susceptibility allele and the C4-C levels. We compared the concentration of C4-C as well as the ratios of C4-C to free carnitine (C0-C) in blood spots of newborns, who are homozygous, heterozygous, or non-carriers for the variant allele and found no significant differences.

Material and methods

1036 newborn screening cards, proportionally obtained from the 14 Dutch screening districts, were screened for the c.625G>A variant, after approval by the Dutch health authorities. DNA was extracted from blood spots by using Chelex (BioRad) essentially as described before. The extracted DNA was subjected to polymerase chain reaction
(PCR)-RFLP analysis to determine the presence of the c.625G>A ACADS variant. The c.625G>A variant creates a recognition sequence for the restriction enzyme Ddel. For the PCR-RFLP analysis, exon 6 of the ACADS gene is amplified from 10 μL extracted DNA in a 25 μL PCR reaction containing 10 mmol/L Tris/HCl pH 8.4, 50 mmol/L KCl, 1.0 mmol/L MgCl2, 0.01% w/v BSA, 0.2 mmol/L dNTP, 1.5 U Taq polymerase and 0.4 μmol/L of each of the following primers: Forward 5'- TCT GAG AAA ACC ACC CGC CTC TCC TTT CTG -3', Reverse 5'- caggaaacagctatgacc TGA GCA CCA TGG CTA TCT TGA AGC -3'. The reverse primer introduces an additional Ddel restriction site (underlined) and contains an M13-rev extension (small letters), which can be used for characterization of the PCR product by means of fluorescent labeled terminator sequencing. The Ddel site in the reverse primer serves as an internal control for the restriction analysis.

The DNA amplification program started with 2 min of denaturation at 96°C, followed by 5 cycles of 30 s at 96°C, 30 s at 55°C, and 30 s at 72°C, and 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, with a final step of 15 min at 72°C. The amplified product was digested for at least 3 hrs at 37°C after the addition of 1/10 volume buffer M and 5 U Ddel (Roche). The restriction fragments were analyzed on a 2% (w/v) agarose gel by ethidium bromide staining. In the presence of the c.625G variant 2 restriction fragments of 208 and 18 bp will be observed; in the presence of the c.625A variant 3 fragments of 180, 28 and 18 bp will be observed. The non-digested PCR fragment comprises 226 bp.

After screening all 1036 screening cards, 34 blood spots were randomly selected from each group of cards that were found to be homozygous, heterozygous or non-carriers for the c.625G>A variant. From these cards new samples, equivalent to approximately 10 μL of blood, were punched for determination of C4-C and C0-C levels using electrospray tandem mass spectrometry. The acylcarnitines were eluted with methanol and analyzed as their butyl esters using ²H₃-, ²H₃-C₃-, ²H₃-C₈-, and ²H₃-C₁₆-carnitine as internal standards.

Analyses of variance were performed to compare the C4-C levels, the C0-carnitine levels, and the C4:C0-C ratios between the 3 groups.

Results and discussion

Screening of 1036 newborn screening cards revealed a number of 57 (5.5%) homozygotes and 324 (31.3%) heterozygotes for the c.625G>A variant. No significant differences were found between the homozygous, heterozygous and non-carriers group for the C4-C concentrations, the C0-C concentrations or the C4:C0-C ratios (Figure and Table).

The total numbers of cards obtained from each of the 14 screening areas were proportional to the number of life births in each of these regions, which guarantees a demographic representation of the population. The percentage of homozygosity we found is comparable to the percentages reported by Corydon and co-workers and...
Kristensen and co-workers, who analysed smaller numbers and found percentages of 4.4, 6.9 and 9.5 in the DNA material of 90 Danish, 102 German, and 95 Spanish individuals, respectively.6;10 Our numbers also correspond well with a study on the frequency of this variant allele in the USA by Nagan and co-workers, who found a percentage of 6% in 694 screening samples.9 Although the analysed DNA samples in these previously reported studies were not proportionally obtained from different demographic areas, potentially introducing a selection bias, the obtained results are similar to ours.

**Table.** Mean and range of C₄⁻ and free (C₀⁻) carnitine levels (μmol/L) and the subsequent C₄⁻:C₀⁻ carnitine ratios in newborn screening cards homozygous, heterozygous, and of non-carriers for the c.625G>A ACADS variant.

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<tr>
<td>Range</td>
<td>0.04-0.46</td>
<td>0.06-0.25</td>
<td>0.06-0.23</td>
</tr>
<tr>
<td>Mean C0-Carnitine (μmol/L)</td>
<td>39</td>
<td>37</td>
<td>38</td>
</tr>
<tr>
<td>Range</td>
<td>19-68</td>
<td>23-65</td>
<td>22-59</td>
</tr>
<tr>
<td>Mean C4:C0-carnitine ratio</td>
<td>0.0042</td>
<td>0.0038</td>
<td>0.0042</td>
</tr>
<tr>
<td>Range</td>
<td>0.0009-0.0083</td>
<td>0.0017-0.0079</td>
<td>0.0025-0.0102</td>
</tr>
</tbody>
</table>

Normal range C₄⁻Carnitine: 0.14-0.87 μmol/L. Normal range C₀⁻Carnitine: 24.7-66.6 μmol/L.

**Figure.** C₄⁻Carnitine levels in bloodspots, homozygous, heterozygous, and of non-carriers for the c.625G>A ACADS variant.
Since clinical signs and symptoms reported in patients with SCADD are variable, ranging from asymptomatic to symptomatic with hypotonia, retardation, epilepsy and ketotic hypoglycaemia, the observed high frequency of this gene variant may have important implications for a potential involvement of SCADD in the pathogenesis of relatively common disorders. However, as 1 in 20 individuals is homozygous for the gene variant and in general probably remains clinically fully asymptomatic, other factors, such as environmental or genetic, may be needed to make the "susceptibility" turn out into clinical disease. Gregersen and co-workers recently suggested a role of chaperone-assisted folding and quality control in this regard.\textsuperscript{1,13-15} Another explanation for the discrepancy between the high prevalence of the gene variant and the low number of symptomatic persons could be that homozygosity for the gene variant represents a non-disease, only predisposing to certain biochemical characteristics but not to clinical symptoms. This would imply that finding homozygosity for the c.625G>A variant together with "SCADD-like symptoms" is based on coincidence only. As "SCADD-like symptoms" are common among patients studied for a potential inborn error of metabolism, and since homozygosity for the gene variant is rather common, a false positive correlation between these two entities can easily be made.

Although the mean C4-C level in the homozygous c.625G>A group was found to be slightly higher than in the other groups, the observed differences were not significant (Figure and Table). To rule out the possible influence of differences in free carnitine levels on the C4-C levels, we also compared the C4:C0-C ratios between these groups. No significant differences were found between the mean C0-C levels or the C4:C0-C ratios (Table). The range of C4-C in the homozygous group was within the normal reference range of our laboratory (0.14-0.87 $\mu$mol/L), but contained 4 bloodspots with C4-C level higher than the concentrations in the non-carriers group. However, the C0 concentrations in these bloodspots were found to be relatively high, so C4:C0 ratios were not higher compared to the non-carriers group.

Our observations are in contrast to the results of Nagan and co-workers, who found the C4-C in blood spots to be significantly higher in subjects homozygous for the c.625G>A variant, although the C4-C levels in their study also never reached the upper limit (1.0 $\mu$mol/L) of their reference range.\textsuperscript{9} Unfortunately, the C4:C0-C ratios were not included in their study. The discrepancy between the results of the study by Nagan and co-workers and our study might be caused by differences in the screening programs performed in the 2 countries. In the Netherlands, newborns are screened between the 5th and 8th day of life. At this time most babies have an adequate caloric intake and have already started to gain weight. Fatty acid oxidation (FAO) is therefore probably suppressed which may result in the absence of C4-C elevation. In contrast, screening in the U.S.A. is already performed at the second day of life, when newborns are generally in a more catabolic state, as adequate caloric intake is not generally achieved yet, resulting in an activated FAO. If this is indeed the cause of the differences between our study and the study by Nagan and co-workers, 2 issues need to be addressed. Firstly it would imply that the
c.625G>A variant indeed affects the expression of the protein and predisposes to higher C4-C levels with potential clinical implications. Secondly, it would have an important implication for the screening for FAO disorders in general in our country, as screening at the end of the first week of life might just be too late to detect patients with FAO disorders.

In conclusion, our study demonstrates a high frequency of the c.625G>A ACADS variant in the Dutch population, which does however not lead to significantly increased C4-C levels in blood spots taken between the 5th and 8th day of life. This latter observation might be the result of the relatively late timing of neonatal screening in our country which would imply that FAO disorders may be missed as a consequence of this late screening. If the c.625G>A variant is indeed associated with clinical SCADD, the high frequency of the variant suggests a possible involvement of SCADD in the pathogenesis of common disorders, probably in relation to other genetic and/or environmental factors. However, homozygosity for the c.625G>A variant might also be a biochemical phenomenon only, representing a non-disease.
References


7. Gregersen N, Winter VS, Corydon MJ et al. Identification of four new mutations in the short-chain acyl-CoA dehydrogenase (SCAD) gene in two patients: one of the variant alleles, S11C-->T, is present at an unexpectedly high frequency in the general population, as was the case for 625G-->A, together conferring susceptibility to ethylmalonic aciduria. *Hum Mol Genet* 1998;7:619-627.


Clinical, biochemical, and genetic heterogeneity in short-chain acyl-CoA dehydrogenase deficiency: implications for newborn screening

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Abstract

Context Short-chain acyl-CoA dehydrogenase (SCAD) deficiency (SCADD) is an autosomal recessive, clinically heterogeneous disorder with only 22 case reports published so far. Screening for SCADD is included in expanded newborn screening programs in most U.S. and Australian states.

Objectives To describe the genetic, biochemical, and clinical characteristics of SCADD patients in the Netherlands and their SCADD relatives, and to explore the genotype to phenotype relation.

Design, setting, and participants Retrospective study involving 31 Dutch SCADD patients diagnosed between 1987 and 2006 and 8 SCADD relatives. SCADD was defined by the presence of 1) increased C4-carnitine (C4-C) in plasma and/or increased ethylmalonic acid (EMA) in urine under non-stressed conditions on at least two occasions, in combination with 2) a mutation and/or the c.511C>T or c.625G>A susceptibility variants on each SCAD-encoding (ACADS) allele. Patients were included only if the ACADS gene was fully sequenced and if current clinical information could be obtained. Relatives were included when they carried the same ACADS genotype as the proband, and had increased C4-C and/or EMA.

Main Outcome Measures Prevalence, genotype (mutation/mutation, mutation/variant, variant/variant), C4-C and EMA levels, clinical signs and symptoms, and clinical course.

Results A birth-prevalence of at least 1:50 000 was calculated. Most patients presented before the age of 3 years, with non-specific, generally uncomplicated, and often transient symptoms. Developmental delay, epilepsy, behavioral disturbances, and hypoglycaemia were the most frequently reported symptoms. The ACADS genotype showed a statistically significant association with EMA and C4-C levels, but not with clinical characteristics. Seven out of 8 SCADD relatives were free of symptoms.

Conclusions SCADD is far more common than assumed previously, and clinical symptoms in SCADD are non-specific, generally uncomplicated, often transient, and not correlated with specific ACADS genotypes. Because SCADD does not meet major newborn screening criteria, including a lack of clinical significance in many patients and that it is not possible to differentiate diseased and non-diseased individuals, it is not suited for inclusion in newborn screening programs at the present time.
Introduction

Short-chain acyl-CoA dehydrogenase (SCAD; MIM 606885) deficiency (SCADD; MIM 201470) is an autosomal recessive inborn error of mitochondrial fatty acid β-oxidation, presenting with a variety of clinical signs and symptoms. Developmental delay, hyper- and hypotonia, ketotic hypoglycemia, and epilepsy are most frequently reported.1-9 SCAD is 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, its substrate (C4-CoA) will accumulate. C4-CoA can be 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). Butyrylcarnitine can be measured in blood, whereas butyrylglycine and EMA can be measured in urine. The latter 3 metabolites may all be elevated in SCADD, although to different extents.

The diagnosis of SCADD may be confirmed by enzyme activity measurements in muscle, fibroblasts, and/or lymphocytes, and by DNA studies.1-6,8-13 Enzyme analysis in fibroblasts and lymphocytes, however, demonstrated a remarkably high residual SCAD activity in several individuals with SCADD.4,10,13 Furthermore, SCAD activity measurements in fibroblasts and lymphocytes gave inconsistent results.6,7 Although the enzyme assay might be more reliable when performed in muscle biopsy specimens,10 this approach is generally considered too invasive for routine testing for SCADD. As consistent and significant low enzyme activity will probably be found in patients who are homozygous for an inactivating mutation only,10 DNA analysis appears to be preferable to confirm the diagnosis of SCADD.

The SCAD-encoding gene, ACADS, has been localized to the terminal region of the long arm of chromosome 12, spans approximately 13 kb, and consists of 10 exons.14 Until now, 17 different mutations have been reported in patients with SCADD.2,3,5-7,10,11,13,15,16 Two of these mutations, c.625G>A and c.511C>T, are common and generally referred to as gene variants.6,7,17-19 They are, in contrast to polymorphisms, considered to confer susceptibility for clinical disease.6,18 The majority of SCADD patients are homozygotes or compound heterozygotes for 1 or 2 of the common ACADS variants, or for a combination of these ACADS variants with a mutation.2,3,5,7,11,16 These ACADS variants have been found in the general population with a prevalence of homozygosity and heterozygosity of approximately 0.3% and 5.6% for the c.511C>T and 5.5% and 31.3% for the c.625G>A variant respectively.17,18 Assuming Hardy-Weinberg equilibrium, the allele frequency based on the prevalence of homozygotes is 5.5% for the c.511C>T and 23.5% for the c.625G>A variant, respectively. Both ACADS variants are considered to play a modifying role in the pathogenesis of clinical SCADD, by conferring susceptibility for clinical disease.6,20 Corydon et al. postulated that certain genetic, cellular, and environmental factors are involved in reducing the catalytic activity of these variant enzymes below a critical threshold, leading to the onset of clinical symptoms.6 Gregersen et al. suggested a
role for chaperone-assisted folding and quality control in this regard.\textsuperscript{21,22} The presence of only these ACADS variants on both alleles might also represent a non-disease, however, only predisposing to certain biochemical characteristics but not to clinical symptoms.\textsuperscript{18}

SCADD appears to be a rare disorder, because until now only 22 genetically confirmed cases have been reported in the literature.\textsuperscript{2-13,15,19} However, the potential role of the common gene variants in the development of clinical SCADD suggested a higher incidence of SCADD.\textsuperscript{6,18}

Apart from the case report of 1 patient who appeared to benefit from riboflavin therapy\textsuperscript{16}, the efficacy of this or other treatments has, to our knowledge, never been systematically studied in a group of SCADD patients. Riboflavin is the precursor of flavin adenine dinucleotide (FAD), and FAD functions as a cofactor for SCAD.

With the development of electrospray tandem mass spectrometry, many countries worldwide have recently expanded their newborn screening program or are preparing to do so in the near future. In the Netherlands, SCADD is not screened for, but screening for SCADD has become part of newborn screening programs in 35 of 51 states in the United States and most Australian states.\textsuperscript{23-25} However, there is no evidence indicating that early detection of SCADD is clinically useful.\textsuperscript{24} Doubts about the indication for screening for SCADD have been expressed by the Newborn Screening Expert Group of the American College of Medical Genetics, acknowledging the lack of evidence related to the availability of a treatment and a poorly understood natural history.\textsuperscript{26,27} With the evolution of newborn screening, 3 policy documents have influenced newborn screening programs.\textsuperscript{28} The latest one, the selection criteria for screening by Frankenburg,\textsuperscript{29} was published in 1974 and provides 8 criteria based on the report by Wilson and Jungner.\textsuperscript{30} The first 3 three criteria state that 1) the disease or condition screened for should be serious or potentially serious, 2) the condition should be relatively common, and 3) it must be possible to differentiate diseased from non-diseased individuals.\textsuperscript{29}

The purpose of our study was to calculate the prevalence of SCADD in the Netherlands and to document and summarize the genetic, biochemical, and clinical characteristics of the largest group of SCADD patients and their SCADD relatives published so far. Within this group, we determined the relation of genotype to biochemical as well as clinical phenotype. In addition, we used the results of our study to discuss newborn screening for SCADD.

Patients and methods

Patients
For this study, SCADD was defined by the presence of 1) increased C4-C level in plasma and/or increased EMA level in urine under non-stressed conditions on at least 2 occasions, in combination with 2) a mutation and/or the c.511C>T or c.625G>A susceptibility variants
on each ACADS allele. Only SCADD patients in whom sequence analysis of all exons and flanking intronic sequences had been performed were included. Patients were included only if clinical information, including development scores and school performance as assessed by the treating physician, could be obtained. Unless a complete recovery was achieved or the patients had died, only patients who had been seen within the last year were included. All 8 metabolic centers in the Netherlands participated in this study. Patients were identified using the SCAD DNA database from the main participating center (Academic Medical Center, Amsterdam), which is the only Dutch center where DNA analysis for SCADD is performed, and by contacting all Dutch metabolic centers. A cross-check on missing patients was performed by consulting the Dutch Diagnosis Registration Metabolic Disorders database, a national registry of all patients diagnosed in the Dutch metabolic centers. This search did not reveal any additional SCADD patients, indicating that the complete cohort of Dutch patients meeting the inclusion criteria were available for the study.

Written informed consent to use anonymous patient information for this study was obtained from the parents and/or legal representatives of all patients participating in this study. The study was reviewed and approved by the Medical Ethics Committee of the Academic Medical Center.

Prevalence Calculation
The number of patients diagnosed from January 2003 until January 2006 was used to calculate the birth-prevalence in the Netherlands. The birth rate used for this calculation was 200 000 per year (Dutch Central Bureau for Statistics).

DNA Analyses
Mutation analysis of the ACADS gene was performed by sequence analysis of all exons and flanking intronic sequences amplified by polymerase chain reaction (PCR) from genomic DNA isolated from either fibroblasts or lymphocytes from the patients. Details on primer composition and PCR conditions are available on request. In case of newly identified mutations, sequence analysis of 100 control alleles was performed to rule out the possibility of a polymorphism. Based on their genotype, we divided the patients into 3 groups: mutation/mutation (mut/mut), mutation/variant (mut/var), and variant/variant (var/var).

Biochemistry
EMA in urine was analyzed by gas chromatography/mass spectrometry of its methoxime/trimethylsilyl derivative as part of the organic acid analyses. It was considered to be increased in cases where the concentrations were 15 μmol/mmol or more of creatinine for children younger than 2 years and 8 μmol/mmol or more of creatinine for children aged 2 years or older.

The level of C4-C in blood was determined as its butyl ester using electrospray tandem mass spectrometry as part of the acylcarnitine analyses. The C4-C concentration was
quantitated by signal comparison with \textsuperscript{2}H\textsubscript{3}-C\textsubscript{3}-carnitine as an internal standard. Reference ranges consisted of the 95th percentile obtained. In this way an upper reference range of 0.58 μmol/L was defined.

Clinical signs and symptoms, patient characteristics, and applied treatment

Information about the patients and the applied treatment was obtained by interviewing the physician of each patient and/or by reviewing the medical charts. Age at first presentation, country of ancestry, symptoms, developmental scores, school performance, clinical course, and applied treatment were summarized. The country of ancestry was used to study potential founder effects. Developmental delay was defined as severe if the IQ was measured or estimated to be <50 or if there was more than 50 % delay in developmental milestones. Epilepsy was defined as severe in the case of persistent seizures that were refractory to drugs. Behavioral disorders were classified as severe when they resulted in the impossibility of attending a regular school or having social contacts. Hypoglycemia was defined as measured blood glucose concentrations of 45 mg/dL (2.5 mmol/L) or less or 47-63 mg/dL (2.6-3.5 mmol/L) in combination with hypoglycemic symptoms, and was classified as severe if glucose concentrations were 27 mg/dL (1.5 mmol/L) or less.

Family studies

Parents of patients seen in one of the participating centers (Academic Medical Center, Amsterdam) were asked to participate in SCAD DNA studies and, if relevant, also to include their other children. The DNA analyses were performed for 37 relatives (20 parents and 17 sibs) of 10 patients. Biochemical analyses were performed for relatives with an \textit{ACADS} genotype identical to the proband. Relatives were labeled as having SCADD when they carried the same \textit{ACADS} genotype as the proband and had increased C4-C and/or EMA. If a parent of a proband was labeled as having SCADD, clinical information was obtained from this parent and, if available, from his/her parents.

Genotype to phenotype relation

Results of biochemical studies were used to explore the relation between genotype and biochemical phenotype. Results of clinical studies were used to explore the relation between genotype and clinical phenotype.

Statistical analysis

The Mann-Whitney test was used to compare genotypes, expressed as an ordinal scale, in patients with and without specific clinical symptoms, i.e. developmental delay, epilepsy, and behavioral disturbances. The Kruskal-Wallis test was used to compare the biochemical results and the percentage of patients with severe, complicated, and uncomplicated symptoms in the 3 different genotype groups. The level of significance
was set at $P<.05$. Analyses were done using Graphpad Prism 3.0 and SPSS 12.0.1 (SPSS Inc, Chicago, Ill) software.

Results

Patients
Thirty-one patients who were diagnosed with SCADD in the Netherlands between January 1987 and January 2006 were included in this study.

Calculated prevalence
The majority of patients ($n = 25$) developed symptoms in the first 3 years of life. Twelve (40%) of the patients included in this study were diagnosed in the Netherlands in the past 3 years. This implies that at least 4 new SCADD patients are born each year in the Netherlands resulting in a birth-prevalence of at least 1:50 000 (95% confidence interval [CI]: 1:29 000-1:87 000).

Genotype
All patients and their genotypes are presented in table 1. In total, 12 different mutations and 2 variants were identified in the Dutch patient group, including 7 mutations that have not been reported previously. Of the newly identified mutations, 5 were missense, 1 was nonsense, and 1 affected splicing. Sequence analysis of 100 control alleles did not reveal any of these mutations. The most common mutation was the c.1058C>T mutation, found in 8 patients of Dutch ancestry (Table 1 and Table 2).

Biochemistry
The EMA values under non-stressed conditions ranged from near normal values to greater than 400 $\mu$mol/mmol of creatinine. The C4-C values ranged from normal values to greater than 4 $\mu$mol/L (Table 1).

Clinical signs and symptoms, patient characteristics, and applied treatment
Patients presented with a variety of clinical signs and symptoms. Most frequently encountered signs and symptoms were developmental delay ($n = 16$), which was non-severe in the majority of patients ($n=15$), epilepsy ($n = 11$, non-severe in all), behavioral disorders ($n = 8$, non-severe in 5), and hypoglycaemia ($n = 6$, non-severe in 5) (Table 2, Figure 1).

Overall, 5 patients (patients 17 and 19 in the mut/var group and patients 23, 25, and 30, var/var group) (Table 2) had one or more severe symptoms. Additional diagnoses, which were considered to be a more likely cause of the signs and symptoms, were made in 2 of them (patients 17 and 23, Table 2). Overall, 7 patients (patients 1 and 2 in the
Table 1. Genotype, EMA, and C4-C in 31 Dutch SCADD patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype*</th>
<th>Age at sample collection</th>
<th>EMA† (µmol/mmol creatinine)</th>
<th>C4-C‡ (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c.815G&gt;A, c.625G&gt;A</td>
<td>0 m</td>
<td>218-330</td>
<td>2.33-4.22</td>
</tr>
<tr>
<td>2</td>
<td>c.988C&gt;T, c.625G&gt;A</td>
<td>7 m</td>
<td>261-414</td>
<td>3.22</td>
</tr>
<tr>
<td>3§</td>
<td>c.1138C&gt;T</td>
<td>2 m</td>
<td>124-380</td>
<td>2.4-4.7</td>
</tr>
<tr>
<td>4</td>
<td>c.IVS1-6C&gt;A, c.625G&gt;A</td>
<td>14 y</td>
<td>13-24</td>
<td>0.68-0.77</td>
</tr>
<tr>
<td>5</td>
<td>c.136C&gt;T, c.625G&gt;A</td>
<td>6 y</td>
<td>61-195</td>
<td>0.97-1.14</td>
</tr>
<tr>
<td>6</td>
<td>c.505A&gt;C, c.625G&gt;A</td>
<td>10 y</td>
<td>17-27</td>
<td>0.5-0.94</td>
</tr>
<tr>
<td>7</td>
<td>c.575C&gt;T, c.625G&gt;A</td>
<td>3 m</td>
<td>44-72</td>
<td>0.96</td>
</tr>
<tr>
<td>8</td>
<td>c.796C&gt;T, c.511C&gt;T</td>
<td>6 m</td>
<td>39-63</td>
<td>0.65-0.72</td>
</tr>
<tr>
<td>9</td>
<td>c.989G&gt;A, c.625G&gt;A</td>
<td>9 m</td>
<td>14-26</td>
<td>0.44</td>
</tr>
<tr>
<td>10</td>
<td>c.989G&gt;A, c.625G&gt;A</td>
<td>3 m</td>
<td>17-30</td>
<td>0.37-0.48</td>
</tr>
<tr>
<td>11</td>
<td>c.1058C&gt;T, c.625G&gt;A</td>
<td>1 y</td>
<td>17-57</td>
<td>1.24-1.58</td>
</tr>
<tr>
<td>12</td>
<td>c.1058C&gt;T, c.625G&gt;A</td>
<td>4 y</td>
<td>35-46</td>
<td>1.41-2.22</td>
</tr>
<tr>
<td>13</td>
<td>c.1058C&gt;T, c.625G&gt;A</td>
<td>1 y</td>
<td>31-80</td>
<td>n.a.</td>
</tr>
<tr>
<td>14</td>
<td>c.1058C&gt;T, c.625G&gt;A</td>
<td>7 y</td>
<td>22-40</td>
<td>0.54-0.71</td>
</tr>
<tr>
<td>15</td>
<td>c.1058C&gt;T, c.625G&gt;A</td>
<td>1 y</td>
<td>9-23</td>
<td>1.22-1.48</td>
</tr>
<tr>
<td>16</td>
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<td>73</td>
<td>0.83</td>
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<tr>
<td>17</td>
<td>c.1058C&gt;T, c.625G&gt;A</td>
<td>1 y</td>
<td>60-110</td>
<td>1.73</td>
</tr>
<tr>
<td>18</td>
<td>c.1058C&gt;T, c.625G&gt;A</td>
<td>1 y</td>
<td>33-122</td>
<td>0.57-1.64</td>
</tr>
</tbody>
</table>

Abbreviations: SCADD, short-chain acyl-CoA dehydrogenase deficiency; ACADS, SCAD-encoding gene; EMA, ethylmalonic acid; C4-C, Butyrylcarnitine; m, months; y, year; n.a., not analysed.

*Gene variants in regular type, mutations in bold type, newly identified mutations underlined.
†Normal range of EMA: 0-2 years: 0-15 µmol/mmol creatinine, ≥2 years: 0-8 µmol/mmol creatinine.
‡Maximum normal concentration of C4-C: <0.58 µmol/L. §Reported previously by Bok et al.10
mut/mut group; patients 14 and 16 in the mut/var group; patients 27, 29, and 31 in the var/var group) had 3 or more different symptoms recorded. An additional diagnosis that was considered to be a more likely cause of the clinical signs and symptoms was made in 2 of them (patients 1 and 16, Table 2). There were 19 patients who had uncomplicated symptoms. Further investigations in one of these patients (patient 18) revealed recurrent urinary tract infections that were due to a subpelvic ureter stenosis, and his symptoms disappeared after surgical intervention. An additional diagnosis, which was more likely the cause of the signs and symptoms, was therefore made in a total of 5 out of 31 patients.

Follow-up after the first presentation ranged from 1 to 18 years. No patients died during follow-up. In 2 patients (both from the var/var group) progressive clinical deterioration was recorded, 12 patients had no change in symptoms during follow-up, 8 patients had amelioration of symptoms, and 9 had complete recovery (Table 2).

Eighteen patients ( Patients 1, 2, 4, 5, 7, 8, 11-15, 17, 19-22, 26, and 27) were treated, in almost all cases temporarily, with riboflavin (vitamin B2). In 6 patients (patients 16, 19, 20, and 25-27), carnitine had been given. In all cases, parents and/or representatives were given instructions to avoid fasting. In 8 patients (patients 8, 10, 11, 21, 23, and 25-27), a cornstarch feed (long-acting carbohydrates) was given before bedtime. No consistent clinical improvement was noted in relation to riboflavin, carnitine, cornstarch, or avoidance of fasting.

Figure 1. Clinical signs and symptoms in 31 Dutch patients with Short-chain acyl-CoA dehydrogenase deficiency. Only symptoms in more than 1 of the total number of 31 patients are included.
Table 2. Clinical signs and symptoms and genotype in 31 Dutch SCADD patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Onset symptoms</th>
<th>Country</th>
<th>Sex/Age</th>
<th>Sex</th>
<th>ACADS mutations on both alleles (mutation/mutation group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1* F/0 d Turkish + + + -</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td>M/1 w</td>
<td></td>
<td>- + + - -</td>
</tr>
<tr>
<td>3†</td>
<td></td>
<td></td>
<td>M/2 m</td>
<td></td>
<td>- - - - -</td>
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</tbody>
</table>

**ACADS mutation on one and ACADS variant on the other allele (mutation/variant group)**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex/Age</th>
<th>Country</th>
<th>Onset symptoms</th>
<th>Sex</th>
<th>ACADS variants on both alleles (variant/variant group)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>F/14 y</td>
<td>Maroc</td>
<td></td>
<td></td>
<td>- - + - -</td>
</tr>
<tr>
<td>5</td>
<td>M/1 y</td>
<td>Dutch</td>
<td></td>
<td></td>
<td>- + - - -</td>
</tr>
<tr>
<td>6</td>
<td>F/7 y</td>
<td>India</td>
<td></td>
<td></td>
<td>- + - - -</td>
</tr>
<tr>
<td>7</td>
<td>F/1 m</td>
<td>Dutch</td>
<td></td>
<td></td>
<td>- - - - -</td>
</tr>
<tr>
<td>8</td>
<td>M/9 m</td>
<td>Turkish</td>
<td></td>
<td></td>
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</tr>
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<tr>
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<td></td>
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<tr>
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<td></td>
<td></td>
<td>- - - - -</td>
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<tr>
<td>15</td>
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<td></td>
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<tr>
<td>16‡</td>
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<td>+ + + + -</td>
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<tr>
<td>17§</td>
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<td></td>
<td></td>
<td>+ + + + +</td>
</tr>
<tr>
<td>18¶</td>
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<td>- - - - +</td>
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</table>

Abbreviations: SCADD, short-chain acyl-CoA dehydrogenase deficiency; ACADS, SCAD-encoding gene; m, months; y, year. *Congenital disorder of glycosylation type 1x was diagnosed during follow-up. †Reported previously by Bok et al.10. ‡Angelman syndrome was diagnosed during follow-up. §Mitochondrial disorder of unknown origin was diagnosed during follow-up. ¶Severe. ©Recurrent urinary tract infections due to a subpelvic ureter stenosis was diagnosed during follow-up. #Adrenal insufficiency was diagnosed during follow-up.
<table>
<thead>
<tr>
<th>Other symptoms</th>
<th>Duration of follow-up, y</th>
<th>Outcome</th>
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<tbody>
<tr>
<td>Microcephaly, multiple, dysmorphic features,</td>
<td>6</td>
<td>Ameliorated</td>
</tr>
<tr>
<td>cutis laxa, hypotonia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food refusal, hypertonia</td>
<td>1</td>
<td>Stable</td>
</tr>
<tr>
<td>Transient infantile hepatic dysfunction</td>
<td>7</td>
<td>Normal</td>
</tr>
<tr>
<td>Fatigue</td>
<td>1</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>Ameliorated</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>Normal</td>
</tr>
<tr>
<td>Food refusal, hypotonia</td>
<td>3</td>
<td>Ameliorated</td>
</tr>
<tr>
<td>Mild pulmonary stenosis</td>
<td>8</td>
<td>Stable</td>
</tr>
<tr>
<td>Microcephaly, clubfeet</td>
<td>1</td>
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</tr>
<tr>
<td>Transient hypotonia</td>
<td>4</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Ameliorated</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Ameliorated</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Normal</td>
</tr>
<tr>
<td>Facial dysmorphism, exercise intolerance</td>
<td>8</td>
<td>Ameliorated</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Normal</td>
</tr>
<tr>
<td>Failure to thrive, spasticity</td>
<td>11</td>
<td>Stable</td>
</tr>
<tr>
<td>Dysmorphism, hypotonia</td>
<td>1</td>
<td>Stable</td>
</tr>
<tr>
<td>Failure to thrive</td>
<td>1</td>
<td>Normal</td>
</tr>
<tr>
<td>Vomiting, fatigue</td>
<td>16</td>
<td>Stable</td>
</tr>
<tr>
<td>Vomiting</td>
<td>16</td>
<td>Stable</td>
</tr>
<tr>
<td>Failure to thrive</td>
<td>5</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Normal</td>
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<tr>
<td></td>
<td>4</td>
<td>Stable</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Unfavorable</td>
</tr>
<tr>
<td>Fatigue</td>
<td>9</td>
<td>Stable</td>
</tr>
<tr>
<td>Hypertonia</td>
<td>9</td>
<td>Ameliorated</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Stable</td>
</tr>
<tr>
<td>Dysmorphic features, scoliosis</td>
<td>14</td>
<td>Stable</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Unfavorable</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Ameliorated</td>
</tr>
</tbody>
</table>

Abbreviations: SCADD, short-chain acyl-CoA dehydrogenase deficiency; ACADS, SCAD-encoding gene; m, months; y, year. *Congenital disorder of glycosylation type 1x was diagnosed during follow-up. † Reported previously by Bok et al.10. ‡ Angelman syndrome was diagnosed during follow-up. § Mitochondrial disorder of unknown origin was diagnosed during follow-up. $ Severe. ¶ Recurrent urinary tract infections due to a subpelvic ureter stenosis was diagnosed during follow-up. # Adrenal insufficiency was diagnosed during follow-up.
Family studies

Of the 37 relatives (20 parents and 17 siblings) tested, 9 relatives (a parent of patients 4, 5, and 21 and a sibling of patients 2, 3, 5, and 21, and two siblings of patient 14) were found to have an ACADS genotype identical to the proband. Except for the father of patient 21 (var/var group) all relatives were found to have increased levels of C4-C and/or EMA (Table 3). Eight of the 9 relatives had no clinical symptoms. The only one with symptoms was a sibling of patient 3 (mut/mut group) who had transient food refusal in her first year of life.

Genotype to phenotype relation

EMA and C4-C values were highest in the 3 patients in the mut/mut group, less increased in the mut/var group, and lowest values were found in the patients with gene variants

Table 3. Genotype, EMA, C4-C, and clinical signs and symptoms in 6 SCADD patients and 9 relatives with an ACADS genotype identical to the proband

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genotype*</th>
<th>Age at sample collection</th>
<th>EMA† (µmol/mmol creatinine)</th>
<th>C4-C‡ (µmol/L)</th>
<th>Clinical signs and symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 2</td>
<td>c.988C&gt;T c.625G&gt;A</td>
<td>7 m</td>
<td>261-414</td>
<td>3.22</td>
<td>Epilepsy, food refusal, hypertonia</td>
</tr>
<tr>
<td>Sibling 2</td>
<td>c.1147C&gt;T</td>
<td>5 y</td>
<td>87</td>
<td>4.05</td>
<td>Transient feeding problems</td>
</tr>
<tr>
<td>Patient 3§</td>
<td>c.1138C&gt;T c.1138C&gt;T</td>
<td>2 m</td>
<td>124-380</td>
<td>2.4-4.7</td>
<td>Transient infantile hepatic dysfunction</td>
</tr>
<tr>
<td>Sibling 3§</td>
<td>c.1138C&gt;T c.1138C&gt;T</td>
<td>15 y</td>
<td>25-58</td>
<td>2.0-6.25</td>
<td>No</td>
</tr>
<tr>
<td>Patient 4</td>
<td>c.IVS1-6C&gt;A c.625G&gt;A</td>
<td>14 y</td>
<td>13-24</td>
<td>0.68-0.77</td>
<td>Fatigue</td>
</tr>
<tr>
<td>Parent 4</td>
<td></td>
<td>45 y</td>
<td>n.a.</td>
<td>0.74</td>
<td>No</td>
</tr>
<tr>
<td>Patient 5</td>
<td>c.136C&gt;T c.625G&gt;A</td>
<td>6 y</td>
<td>61-195</td>
<td>0.97-1.14</td>
<td>Developmental delay, epilepsy</td>
</tr>
<tr>
<td>Parent 5</td>
<td></td>
<td>43 y</td>
<td>19</td>
<td>1.14</td>
<td>No</td>
</tr>
<tr>
<td>Sibling 5</td>
<td></td>
<td>13 y</td>
<td>26</td>
<td>0.94</td>
<td>No</td>
</tr>
<tr>
<td>Patient 14</td>
<td>c.1058C&gt;T c.625G&gt;A</td>
<td>7 y</td>
<td>22-40</td>
<td>0.54-0.71</td>
<td>Developmental delay, facial dysmorphism</td>
</tr>
<tr>
<td>Sibling 14-1</td>
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<td>6 y</td>
<td>17-18</td>
<td>n.a.</td>
<td>No</td>
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<tr>
<td>Sibling 14-2</td>
<td></td>
<td>6 y</td>
<td>17-21</td>
<td>n.a.</td>
<td>No</td>
</tr>
<tr>
<td>Patient 21</td>
<td>c.625G&gt;A c.625G&gt;A</td>
<td>1 y</td>
<td>16-20</td>
<td>0.43-0.64</td>
<td>Hypoglycemia</td>
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<tr>
<td>Parent 21</td>
<td></td>
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<td>4-5</td>
<td>0.36-0.48</td>
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<tr>
<td>Sibling 21</td>
<td></td>
<td>5 y</td>
<td>7-18</td>
<td>0.53</td>
<td>No</td>
</tr>
</tbody>
</table>

Abbreviations: EMA, ethylmalonic acid; C4-C, Butyrylcarnitine; SCADD, short-chain acyl-CoA dehydrogenase deficiency; ACADS, SCAD-encoding gene; m, months; n.a., not analysed. *Gene variants in regular type and mutations in bold type. †Normal range of EMA: 0-2 years: 0-15 µmol/mmol creatinine, ≥2 years: 0-8 µmol/mmol creatinine. ‡Maximum normal concentration of C4-C: <0.58 µmol/L. §Reported previously by Bok et al.10
only. A statistically significant association between EMA as well as C4-C concentrations and the genotype ($P=.002$ in both) was detected (Figure 2).

The genotype of the SCADD patients and SCADD relatives was not related to the nature or the severity of symptoms (Table 3 and Figure 3).

**Figure 2.** Correlation of genotype to mean ethylmalonic acid and butyrylcarnitine in 31 Dutch patients with short-chain acyl-CoA dehydrogenase deficiency. For both ethylmalonic acid and C4-carnitine, there is a significant difference ($P=.002$) between the 3 genotype groups. Mut indicates mutation; var, variant.

**Figure 3.** Correlation of genotype to the nature and the severity of symptoms in 31 Dutch patients with short-chain acyl-CoA dehydrogenase deficiency. Symptoms were defined as severe in the case of 1 or more severe symptoms, complicated in the case of 3 or more non-severe symptoms, and uncomplicated in the case of 2 or fewer non-severe symptoms. For nature of symptoms, there were no significant differences ($P=.29$ for developmental delay, $P=.78$ for epilepsy, $P=.05$ for behavioral disturbances) among the 3 genotype groups. For severity of symptoms, there was also no significant difference ($P=.22$) among the 3 genotype groups. Mut indicates mutation; var, variant.
Comment

Our study presents the largest group of SCADD patients reported so far. As extended diagnostic studies are more likely to be performed and clinical information is more likely to be available in the case of severe symptoms, our study population might represent the more severely affected SCADD patients.

Based on the number of patients diagnosed in the last 3 years, a birth-prevalence for the Netherlands of at least 1:50 000 was calculated. The true prevalence may well be higher, in view of the strict inclusion criteria used in our study. Furthermore, metabolic studies are generally performed in only a minority of patients with symptoms found in SCADD, such as mild developmental delay and epilepsy. Indeed, results of newborn screening studies performed in the United States indicate a higher prevalence. Birth-prevalences as high as 1:33 000 were estimated for classic SCADD, defined as SCADD with mutations on both alleles and very high C4-C levels as detected by using high cut-off levels (4-10 SD above the mean) for C4-C. In the group of 31 patients reported herein, only 3 (9.7%) were found to have mutations on both alleles of the ACADS gene and are thus comparable to SCADD in its classic form. If one assumes that the prevalence of classic SCADD (mut/mut group) and SCADD with a mut/var or var/var genotype (non-classic SCADD) is similar in the United States and the Netherlands, this would imply that the birth-prevalence of SCADD in the Netherlands, including both forms, might be approximately 10 times as high, which would result in a birth-prevalence of SCADD of approximately 1:3300. This prevalence is even higher than the prevalence of phenylketonuria (PKU), which is assumed to be one of the most common inborn errors of metabolism with a birth-prevalence of 1:10 400 to 1:4500 if all variant forms of PKU are included.

Our study revealed 7 newly identified ACADS mutations, resulting in a total number of 22 different ACADS mutations, apart from the two ACADS variants, published so far (Table 4). The biochemical features of patients with the newly identified missense mutations, combined with the fact that these mutations have not been observed in 100 control alleles, strongly suggests that these are inactivating mutations. The c.1058C>T mutation was found in 8 of the 20 patients with mutations, all of Dutch ancestry, suggesting a founder effect. This mutation has been reported previously, also in a patient of Dutch ancestry.

Clinical signs and symptoms at presentation were highly variable, with developmental delay (non-severe in almost all patients), epilepsy, behavioral disturbances, and hypoglycaemia being the most frequently reported (Table 2 and Figure 1). Most patients presented with more than one of these symptoms. Except for behavioral disorders these symptoms were also frequently noted in patients reported previously.

Most of the severely affected patients belonged to the var/var group (Table 2), which is in line with other studies. The overrepresentation of variant alleles in severely affected SCADD patients may be the result of a selection bias, since it is more likely
that a full metabolic screening is performed in patients with severe clinical symptoms.  

Because approximately 6% of the general population is homozygous or compound heterozygous for the c.625G>A and/or c.511C>T \textit{ACADS} gene variants, the apparent association between clinical symptoms and the presence of variant alleles might be coincidental. Therefore, the detection of homozygosity or compound heterozygosity for these gene variants in patients with severe clinical symptoms should not preclude a full diagnostic workup for other potential causes of the symptoms. Further diagnostic studies in SCADD patients with either a mut/mut or mut/var genotype may also be indicated.

Table 4. Newly identified and previously reported \textit{ACADS} mutations and variants

<table>
<thead>
<tr>
<th>Reference</th>
<th>DNA mutation</th>
<th>Coding effect*</th>
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<tr>
<td><strong>Mutations</strong></td>
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</tr>
<tr>
<td>Current study</td>
<td>IVS1-6C&gt;A</td>
<td>Putative splicing error</td>
</tr>
<tr>
<td>Naito et al.,\textsuperscript{15} 1990</td>
<td>c.136C&gt;T</td>
<td>p.R46W</td>
</tr>
<tr>
<td>Corydon et al.,\textsuperscript{6} 2001</td>
<td>c.268G&gt;A</td>
<td>p.G90S\textsuperscript{†}</td>
</tr>
<tr>
<td>Gregersen et al.,\textsuperscript{7} 1998</td>
<td>c.274G&gt;T</td>
<td>p.G92C\textsuperscript{†}</td>
</tr>
<tr>
<td>Corydon et al.,\textsuperscript{6} 2001</td>
<td>c.310-312 del GAG</td>
<td>p.E104del\textsuperscript{†}</td>
</tr>
<tr>
<td>Naito et al.,\textsuperscript{15} 1990</td>
<td>c.319C&gt;T</td>
<td>p.R107C</td>
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<tr>
<td>Koeberl et al.,\textsuperscript{2} 2003</td>
<td>c.332C&gt;T</td>
<td>p.S111F</td>
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<tr>
<td>Koeberl et al.,\textsuperscript{2} 2003</td>
<td>c.409C&gt;T</td>
<td>p.Q137X</td>
</tr>
<tr>
<td>Seidel et al.,\textsuperscript{13} 2003</td>
<td>c.417G&gt;C</td>
<td>p.T139C\textsuperscript{†}</td>
</tr>
<tr>
<td>Current study</td>
<td>c.505A&gt;C</td>
<td>p.T169P</td>
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<tr>
<td>Gregersen et al.,\textsuperscript{7} 1998</td>
<td>c.529T&gt;C</td>
<td>p.W177R\textsuperscript{†}</td>
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<td>Corydon et al.,\textsuperscript{6} 2001</td>
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<td>p.A192V\textsuperscript{†}</td>
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<td>p.R325W\textsuperscript{†}</td>
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<td>p.R330C</td>
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<td>p.R380W\textsuperscript{†}</td>
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<td>Current study</td>
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<td>p.I390M\textsuperscript{††§}</td>
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<td>Gregersen et al.,\textsuperscript{7} 1998</td>
<td>c.511C&gt;T</td>
<td>p.R171W</td>
</tr>
<tr>
<td>Kristensen et al.,\textsuperscript{34} 1994</td>
<td>c.625G&gt;A</td>
<td>p.G209S</td>
</tr>
</tbody>
</table>

Abbreviations: ACADS, Short-chain acyl-CoA dehydrogenase (SCAD)-encoding gene. *Nomination according to the human genome variation society. †Mutant SCAD protein was found to result in undetectable activity after expression in E. coli or COS-7 cells. ‡DNA analysis performed by Corydon and Gregersen in Aarhus, Denmark. §Expression studies performed by Vockley in Rochester, USA.
The importance of further studies is illustrated by patients 1, 16, 17, and 18 in whom additional diagnoses were made that are more likely to be causing the clinical symptoms. These findings, as well as the results of the family studies reported herein, demonstrate that even missense mutations can occur without any clinical significance.

The clinical course was rather similar in most patients. In general, symptoms developed early in life, which was also reported in previous studies. Complete recovery of symptoms was reported in 9 of the 31 patients reported herein and in 8 of the 10 patients in whom outcome was reported previously, suggesting that in a substantial number of patients, SCADD is associated with transient clinical symptoms. In our study, no consistent improvement was reported in response to riboflavin, carnitine, cornstarch, or avoidance of fasting. However, more studies are necessary to assess the effect of treatment, in particular of riboflavin therapy.

Although a significant association was found between genotype and biochemical phenotype, our study did not reveal an association between genotype and clinical features in SCADD. This finding suggests that modifying factors may be involved in the pathogenesis of clinical SCADD, as previously suggested for the c.625G>A and c.511C>T susceptibility alleles. Our observation that SCADD is often associated with transient clinical symptoms might be related to a temporary nature of these factors. Neurological symptoms may be caused by EMA, which was found to be toxic to neuronal cells, and for free butyrate, which may cause encephalopathy. During circumstances with increased demand on mitochondrial fatty acid oxidation, such as prolonged fasting, concentrations of these potential toxic metabolites may increase, resulting in reversible neurotoxicity.

The relatively benign clinical course observed in many of the SCADD patients implies that SCADD does not meet the first Frankenburg screening criterion stating that the disease or condition screened for should be serious or potentially serious. In addition, 7 of the 8 relatives identified in this study with an ACADS genotype identical to the proband and increased C4C and/or EMA levels, were free of symptoms. This observation also implies that it is not possible to differentiate SCADD patients from non-diseased SCADD individuals. Therefore, SCADD does not meet the third Frankenburg criterion for screening. This is in line with the observation that all 7 individuals detected by newborn screening in Australia with probable SCADD remained free of symptoms without any treatment during subsequent follow-up of 2-7 years (written communication, B. Wilcken, The Children's Hospital at Westmead, Westmead, Australia). Furthermore, 17 putative SCADD patients as well as 2 of the 3 patients with confirmed SCADD based on homozygous mutations, detected by newborn screening programs in the United States, did not develop any clinical symptoms during the first years of life. Although the results of our study suggest that SCADD is relatively common, thus meeting the second Frankenburg criterion, we believe that SCADD should not be included in neonatal screening programs at this time. Indeed, screening for SCADD may have negative consequences placing families at risk for increased stress and parent-child dysfunction.
However, infants already identified by SCADD newborn screening should be included in long-term follow up studies to obtain more information to decide about the relevance of screening for SCADD.

Unfortunately, both the Wilson and Jungner and the Frankenburg criteria have limitations in the context of newborn screening using tandem mass spectrometry. However, no criteria have been published since and they are still applied in the discussion on newborn screening. The results of a new approach for recommending conditions for newborn screening by using an expert panel recently were published by the American College of Medical Genetics. In this report, SCADD was not included in the core panel of diseases for which screening is considered mandatory. SCADD was included in the group of secondary targets because it is in the differential diagnosis of a condition in the core panel and it is of clinical significance. However, the results of our study and newborn screening studies demonstrate that SCADD lacks clear clinical significance in many patients, implying that SCADD should not be included in the group of secondary targets and it does not qualify for newborn screening at this time.

This study has several limitations in addition to the underestimate of prevalence given the use of a clinically rather than screened population. As data were collected retrospectively, no sequential neuropsychological and motor development tests were performed. In addition, clinical information was scored by different physicians. To further elucidate the clinical spectrum of SCADD, more prospective and long-term studies, including formal neuropsychological and motor development testing, are necessary.

In summary, the results of newborn screening studies as well as our data suggest that SCADD is far more common than assumed previously. In the Dutch patient cohort, clinical symptoms are non-specific, generally uncomplicated, often transient and not related to the $ACADS$ genotype. These observations, in combination with the observation that almost all relatives diagnosed with SCADD, as well as almost all individuals found by neonatal screening, remain asymptomatic, suggest that an association between symptoms and SCADD is often spurious. In some individuals carrying $ACADS$ variants and/or mutations, environmental or other genetic factors may result in true SCADD-related clinical pathology. In many other individuals, however, SCADD may only be a lifelong biochemical phenomenon. Because SCADD does not meet major newborn screening criteria, it is not suited for inclusion in newborn screening programs at this time.

Acknowledgments
References


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Chapter 4

Fasting and fat-loading tests provide pathophysiological insight into short-chain acyl-CoA dehydrogenase deficiency

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Abstract

**Objective** To gain insight into the pathophysiological and clinical consequences of short-chain acyl-CoA dehydrogenase deficiency (SCADD).

**Study design** A retrospective study of 15 fasting and 6 fat-loading tests in 15 Dutch patients with SCADD, divided into 3 genotype groups. Metabolic and endocrinologic measurements and the biochemical characteristics of SCADD, ethylmalonic acid (EMA) and C4-carnitine (C4-C) were studied.

**Results** Three patients had development of hypoglycemia during fasting; all of these had originally presented with hypoglycemia. Metabolic and endocrinologic measurements remained normal during all tests. The EMA excretion increased in response to fasting and fat-loading, and plasma C4-C remained stable. Test results did not differ between the 3 genotype groups.

**Conclusions** The metabolic profiles of the 3 patients with development of hypoglycemia resemble idiopathic ketotic hypoglycemia. Because hypoglycemia generally requires a metabolic work-up and because SCADD is relatively prevalent, SCADD may well be diagnosed coincidently, thus being causally unrelated to the hypoglycemia. If SCADD has any other pathological consequences, the accumulation of potentially toxic metabolites such as EMA is most likely involved. However, the results of our study indicate that there is no clear pathophysiological significance, irrespective of genotype, supporting the claim that SCADD is not suited for inclusion in newborn screening programs.
Introduction

Short-chain acyl-CoA dehydrogenase deficiency (SCADD) is an autosomal recessive inborn error of mitochondrial fatty acid oxidation (FAO). Most patients with SCADD have been diagnosed as a result of investigations for neurological symptoms and/or hypoglycemia. SCADD is screened for in the United States, although it does not meet newborn screening criteria, and there is no evidence that early detection would be clinically useful.

The short-chain acyl-CoA dehydrogenase (SCAD) protein is the first enzyme involved in the short-chain fatty acid β-oxidation spiral. When SCAD activity is impaired, its substrate, butyryl-CoA (C4-CoA), accumulates and is subsequently converted into different metabolites, including butyrylcarnitine (C4-C), butyrate, and ethylmalonic acid (EMA). C4-C can be measured in blood and EMA can be measured in urine.

DNA analysis is the most reliable method to confirm a diagnosis of SCADD. Most patients with SCADD are homozygous or compound heterozygous for 2 common SCAD-encoding gene (ACADS) variants, or for ACADS variants in combination with an inactivating mutation. Homozygosity for ACADS variants has been found in the general population with a remarkably high prevalence, at approximately 0.3% for the c.511C>T and 5.5% for the c.625G>A variant, and is considered to confer susceptibility to development of clinical disease. However, the factors involved in turning this “susceptibility” into clinical symptoms have not yet been elucidated.

Mitochondrial FAO plays an important role in energy production, especially during periods of prolonged fasting. Pathophysiological mechanisms of inborn errors in FAO include the following: (1) inadequate supply of energy; (2) sequestration or loss of vital components of intermediary metabolism; and (3) accumulation of toxic metabolites. Although these metabolic consequences have been partially elucidated for long- and medium-chain FAO disorders, the pathophysiological mechanisms involved in SCADD are less clear.

We carried out fasting and fat-loading tests on patients with SCADD to assess the potential risks of the disorder. This study was designed to critically review the data of 15 fasting and 6 fat-loading tests in order to gain insight into the pathophysiological and potential clinical consequences of SCADD.

Methods

Patients

Fifteen patients were included in this study (Table). All patients were initially investigated because of clinical symptoms (4 patients because of a hypoglycemic episode). They were subsequently diagnosed with SCADD on the basis of increased C4-C in plasma and/or
increased EMA in urine under non-stressed conditions on at least 2 occasions, along with
the presence of a mutation and/or the c.511C>T or c.625G>A variants on each ACADS
allele. Patients were classified in 3 different groups on the basis of their ACADS
genotype, which include the following: ACADS mutations on both alleles (mutation/mutation [mut/
mut] group; n=3); an ACADS mutation on 1 and an ACADS variant on the other allele or
both alleles (mutation/variant [mut/var] group; n=8); and ACADS variants on both alleles
(variant/variant [var/var] group; n=4) (Table). All patients were part of the Dutch SCADD
cohort that was previously described.\(^2\)

Tests
Fasting tests were performed in all patients except for patient 1, for whom parental
consent was not obtained. Fat-loading tests were only performed in the first 6 presenting
patients (patients 1, 4-7, and 13) and none thereafter because these tests appeared not
to provide additional information beyond the fasting test data. All tests were performed
between January 2001 and January 2008. Written informed consent was obtained from
the parents or legal representatives of all participating patients. This study was reviewed
and approved by the Medical Ethics Committee of the Academic Medical Center.

Fasting protocol
Patients were admitted to our hospital 1 day before the test and only fasted if food
intake during three days prior to admission had been normal and only if they were well.
During the test, patients were continuously observed by experienced staff. The maximum
duration of the fast was determined by the age of the child. Patient 11 (13 years old)
fasted for a maximum of 46 hours. All other patients (1 to 8 years old) fasted for a
maximum of 22 hours. The fast was terminated earlier if the child showed clinical signs
of hypoglycemia or if a blood glucose (BG) level \( \leq 45 \text{ mg/dL} \) (2.5 mmol/L) was measured.
In the case of the 22-hour fasting test, fasting started after a meal at 18:00 hours. BG
was monitored every 2 hours after the first missed meal and every hour after 19 hours
of fasting. The measurement frequency was increased if BG concentrations fell rapidly
or if there were clinical concerns. Blood samples for the measurement of metabolites
and hormone levels were taken after 15 and 19 hours and at the end of the test. Blood
samples were drawn using intravenous access while avoiding cuffing. Urine for organic
acid analysis was collected from 0-12 hours of fasting, from 12 hours fasting until the
end of the test, and from the end of the test until a urine sample of at least 50 mL was
obtained. In the case of the 46-hour fasting test, the above blood and urine collection
schedules were adjusted.

Fat-loading protocol
Preparations were similar to those used in the fasting test. On the day of admission,
patients fasted from 21:00 hours onward. Twelve hours later (t=0) 1.5 mL/kg (maximum
of 50 mL) of sunflower oil was administered either orally or through a nasogastric
tube. The sunflower oil was purchased from the regular hospital supplier. Its fatty acid composition was essentially oleic (21%) and linoleic (63%) acids. Blood samples for the determination of metabolite concentrations were obtained at 0, 60, 90, 120, 180, 240, and 360 minutes after fat-loading. Urine was collected in 3 portions: from 9 hours before fat-loading until fat-loading, from fat-loading until 6 hours after fat-loading, and from 6 hours to 10 hours after fat-loading.

Blood and urine analysis

Blood samples were analyzed for BG, nonesterified fatty acids (NEFA) and ammonia levels in both tests. Endocrinologic measurements (insulin, c-peptide, growth hormone, and cortisol) were examined in the fasting tests only. In addition, biochemical analyses of blood samples were performed to explore lactate, pyruvate, ketone bodies (KB), and acylcarnitine profiles in both tests, and amino acids profiles were assessed in the fasting tests. Acylcarnitine profiles were by use of electrospray tandem mass spectrometry. Blood samples for lactate, pyruvate, and KB were immediately deproteinized with perchloric acid and stored on ice, followed by quantitative determination of metabolites with standard spectrophotometric or fluorimetric methods. Blood samples for determination of ammonia levels were immediately stored on ice. All samples were delivered to the laboratory within 10 minutes of collection. Urine samples were analyzed for organic acids by gas chromatography/mass spectrometry, and stored at -20°C until analysis.

Interpretation of test results

For the purposes of the tests described, hypoglycemia was defined as a BG level ≤45 mg/dL (2.5 mmol/L) or 47-54 mg/dL (2.6-3.0 mmol/L) in combination with hypoglycemic symptoms (decreased consciousness and/or vegetative symptoms). KB concentrations during fasting were considered subnormal if the total blood KB concentration was <1.8 mmol/L and abnormal if <0.8 mmol/L, both in the presence of a BG <54 mg/dL (3.0 mmol/L). KB concentrations at the end of the fasting test were also considered abnormal in the case of a NEFA/KB ratio <2.6. The KB response to fat-loading was defined as abnormal if the KB concentration increased by less than twice its initial (t=0) concentration. KB response after fasting and fat-loading was considered abnormal if no statistically significant decrease in the FFA/KB ratio was observed. During hypoglycemia onset, patients were considered to have hyperinsulinism if plasma insulin levels were >15 pmol/L, hypocortisolism if plasma cortisol levels were <400 nmol/L, and growth hormone deficiency if growth hormone levels were <15 mE/L.

Statistical analysis

The Kruskal-Wallis test was used to compare the 3 different genotype (mut/mut, mut/var, and var/var) groups and 3 or more different periods. The Wilcoxon matched pairs signed ranked sum test was used to compare baseline values with values from the end of the tests. The level of significance was set at P <.05. Analyses were performed with
Graphpad Prism 3.0 (Graphpad Software, San Diego, California) and SPSS 12.0.1 (SPSS, Chicago, Illinois) software.

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Results

Patients

The genotypes and clinical phenotypes of the participating patients are shown in the Table.

Fasting tests

One fasting test was terminated prematurely because of possible hypoglycemic symptoms that were not confirmed by BG measurement (patient 2, Table). Hypoglycemia developed in 3 patients during fasting (Table). In these patients, insulin levels were appropriately

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Pt, patient; *Age at time of test; †Gene variants in regular type, mutations in bold type
decreased, and growth hormone and cortisol levels were appropriately increased by the end of the test. Ammonia, lactate, pyruvate, and amino acid levels remained normal during all tests. To assess the need for continuation of a late evening feeding, patient 12 underwent a second fasting test 2 years after the first one. KB production was subnormal during the first fasting test of patient 12 (Figure 1, A). In all other fasting tests, including the second fasting test in patient 12, KB production was within the normal reference range (Figure 1). No statistically significant differences were observed in KB production between the mut/mut, mut/var, and var/var groups ($P = .24$ for glucose x KB and $P = .34$ for NEFA/KB; Figure 1).

The plasma free carnitine concentration decreased during fasting in all patients, but remained within the normal range (22.3-54.8 $\mu$mol/L). The concentrations of C4-C, as well as the C4-C/free carnitine ratios, did not significantly change during fasting in the mut/var ($P = .27$ and $P = .65$, respectively) and var/var ($P = .75$ and $P = .28$, respectively) groups (Figures 2, A and B). They were significantly different between the 3 genotype groups ($P = .027$ for both), with the highest levels in the mut/mut and the lowest in the

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<th>Clinical signs and symptoms</th>
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var/var group (Figures 2, A and B). The EMA levels increased in a statistically significant manner in the mut/var (P = .025) and var/var (P = .04) groups (Figure 2, C).

Fat-loading tests
Two of the 6 fat-loading tests (in patients 1 and 6) were terminated prematurely (after 3 and 4 hours, respectively) because of a loss of intravenous access. BG and ammonia levels were normal during all tests. KB production was sufficient in all patients with a statistically significant, on average approximately 6-fold, increase of KB levels (P = .03; Figure 3, A), and a statistically significant decrease of the ratio of NEFA to KB (P = .01; Figure 3, B) over the course of measurement. C4-C values did not change significantly during the test (P = .73; Figure 3, C). EMA excretion increased during the test; the change in excretion was not statistically significant (P = .27; Figure 3, D).

Discussion
Fasting-induced hypoglycemia has been reported in several patients with SCADD and was the presenting symptom in 19% of the Dutch patients previously described. However, it was not known whether all patients with SCADD are at risk for fasting-induced hypoglycemia. Moreover, a true causal relationship between SCADD and hypoglycemia has not been established. Our study indicates that only those patients with SCADD who had a history of hypoglycemia had development of hypoglycemia during a fasting test,
Figure 2. Biochemical characteristics of 14 patients with SCADD, subdivided into mut/mut, mut/var and var/var genotype groups, during fasting. In the 46-h fasting test the T=36 result is stated at T=19. A, C4-C; the dashed line represents the upper limit of the normal range (0.58 μmol/L). B, C4-C/free carnitine ratios. C, EMA levels; the dashed line represents the upper limit of the normal range (8 μmol/mmol creatinine).

whereas 11 out of our 14 patients (79%) did not have development of hypoglycemia. Therefore diagnosing SCADD should not routinely be followed by a fasting test or the introduction of a late evening meal, unless the clinical history suggests hypoglycemia.

Apart from the biochemical characteristics of SCADD (increased C4-C in plasma and/or increased EMA in urine, as observed in all patients), metabolic evaluation failed to detect any other metabolic or endocrine abnormalities in patients with development of hypoglycemia. Therefore, hyperinsulinism does not contribute to hypoglycemia in SCADD, as it does in short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency, another short-chain FAO disorder.20 The observed ketotic hypoglycemia in the 3 SCADD patients resembled idiopathic ketotic hypoglycemia (IKH or “toddlers hypoglycemia”),21, 22 the most common cause of hypoglycemia in children, which is caused by an inability to maintain adequate glucose production levels.21 IKH is a diagnosis of exclusion, and this diagnosis can be made in hypoglycemic patients only when proper investigations fail to
detect a cause for the observed hypoglycemia. The high frequency of IKH among the SCADD patients group (3 of 14) could be due to ascertainment bias as a metabolic workup, which might lead to the diagnosis of SCADD, is normally requested in all patients presenting with hypoglycemia. Because the c.625G>A ACADS variant is common in the general population (5.5% homozygosity in the Netherlands), and the c.1058C>T mutation has been found to be the most common Dutch ACADS mutation, a diagnosis of SCADD due to this ACADS variant and mutation is relatively prevalent and could well be unrelated to the hypoglycemia in these patients. Indeed, we have recently suggested

**Figure 3.** Ketogenesis expressed as A, KB production and B, the ratio of NEFA to KB and biochemical characteristics of SCADD; C, C4-C and D, EMA in response to fat-loading in 6 patients with SCADD. The dashed lines represent the upper limit of normal levels: 0.58 µmol/L for C4-C and 8 µmol/mmol creatinine for EMA.
that, for at least some patients, SCADD might be a life-long biochemical phenomenon that is not correlated with clinical disease. Our observation that only 3 of the 14 SCADD patients had development of a ketotic hypoglycemia during fasting, and only those who had a prior hypoglycemia history, supports this claim. The observation that patient 12 did not have development of hypoglycemia during a second fasting test 2 years later might be considered an indication for transient disease in SCADD, as has been previously suggested. However, this observation also fits the transient nature of IKH.

The normal ketotic response observed during the fasting tests in all 14 patients with SCADD is further substantiated by the normal ketotic response observed during the 6 fat-loading tests. Long chain (C16-C20) fatty acids are the predominant substrates for FAO during fasting, comprising most of the fatty acids present in sunflower oil, and can undergo 6 to 8 FAO cycles, resulting in 6 to 8 molecules of acetyl-CoA before the decreased activity of SCAD impairs the final \( \beta \)-oxidative cleavage of C4-CoA. This final step in \( \beta \)-oxidation would normally result in the production of 2 additional acetyl-CoA units. Apparently, the ability to produce at least (depending on residual SCAD activity) 75% of the normal amount of acetyl-CoA units, and thus at least 75% of normal KB production, is sufficient for a normal ketogenic response under fasting conditions and after fat-loading. It also appears to be sufficient for adequate formation of N-acetylglutamate, which activates the first enzyme of the urea cycle, because ammonia levels did not increase (in contrast to medium-chain acyl-CoA dehydrogenase deficiency patients) during hypoglycemia.

Hypoglycemia caused by disorders of mitochondrial FAO is believed to be caused by a reduction in gluconeogenesis in combination with a decrease in the glucose-sparing effect of ketone bodies. However, in 11 out of 14 patients with SCADD, fasting was well tolerated, and normoglycemia was sustained. In addition, the concentrations of lactate and alanine, which are both precursors of gluconeogenesis, did not increase during fasting, (data not shown), suggesting normal gluconeogenesis. Furthermore, ketogenesis was adequate in all patients. Therefore the results of our study do not provide evidence for a role of inadequate energy supply in the pathophysiology of SCADD.

Free carnitine levels decreased in patients with SCADD during fasting, comparable with levels in control subjects, but remained within the normal range. These findings suggest that sequestration or loss of carnitine is also not involved in the pathophysiology of SCADD.

A statistically significant increase in EMA excretion was detected in all 3 genotype groups during fasting. In addition, a clear, but not statistically significant increase was observed after fat-loading. This increase in EMA excretion is likely due to the accumulation of C4-CoA, the substrate of SCAD, which is converted into ethylmalonyl-CoA by propionyl-CoA carboxylase and subsequently into ethylmalonic acid by one of the mitochondrial acyl-CoA hydrolases. Remarkably, levels of C4-C, as produced from C4-CoA by the mitochondrial enzyme carnitine acetyltransferase, did not increase in the plasma of patients with SCADD, which suggests a preference for the EMA pathway during fasting and fat-loading. The observation that the activation of FAO by fasting
and fat-loading results in an increase of EMA suggests that if SCADD is associated with any pathophysiological effect, the accumulation of metabolites like EMA is most likely involved in the pathophysiology of SCADD. EMA inhibits the activity of mitochondrial CK at 1.0 mmol/L in rat brain slices. It is, however, questionable whether concentrations this high will occur in the brains of patients with SCADD. Butyrate, another metabolite, which might accumulate as a result of SCADD, has been suggested to be cytotoxic to the human brain, however, no studies have been conducted to demonstrate this effect. Finally, if accumulation of EMA and C4-C contributes to the pathophysiology of SCADD in vivo, it would be expected that patients with the highest levels of these metabolites be the most severely affected. However, we demonstrated here that patients with the highest levels of EMA and C4-C (the mut/mut genotype group) were not more severely affected with respect to ketogenesis when compared to patients with lower EMA and C4-C levels (the mut/var or var/var genotype groups). Furthermore, in our previous study\(^2\) and in the study of Pedersen et al,\(^3\) similar observations were made with respect to clinical symptoms. Therefore the question remains as to whether EMA, C4-C, and/or other metabolites derived from butyryl-CoA, such as butyrate, are related to the clinical signs and symptoms of patients with SCADD.

SCADD is part of newborn screening programs in the majority of states in the United States.\(^27\) However, we recently suggested that SCADD is not suited for inclusion in newborn screening programs because it lacks clear clinical significance in many patients and does not meet major newborn screening criteria.\(^2\) Australian screening programs therefore no longer include SCADD in their screening panel.\(^28\) The normal metabolic responses observed in this study and the absence of a direct relationship between accumulating metabolites and clinical symptoms in patients with SCADD support the clinical irrelevance of SCADD.

Inadequate supply of energy, as reflected by impaired ketogenesis or gluconeogenesis, or loss of vital components of intermediary metabolism, such as carnitine, does not appear to be relevant in SCADD. Because the level of EMA increased significantly during fasting and fat-loading, the accumulation of potentially toxic metabolites is more likely to be involved in the pathophysiology of SCADD. Because this hypothesis is not sufficiently supported by experimental data, further research is necessary to explore whether these metabolites have pathophysiological effects in SCADD.

**Acknowledgments**

This work was supported by a research grant from the Dutch association for research on metabolic disorders “Metakids” (www.metakids.nl). We thank the patients with SCADD and their parents for participation in this study and are grateful to Thessa Westphal and Klaske Honig for their skillful assistance during all performed tests. In addition, we thank J.H. van der Lee of the Center for Pediatric Clinical Epidemiology for assistance on statistical analyses.
References


Chapter 5

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 C4-Carnitine (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, co-factor), 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 SCADD patients, 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 as 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 4 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.
Introduction

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, hypoglycemia, and hypotonia, but the diagnosis of SCADD probably has no clinical significance in many individuals. SCADD is one of the more common inborn errors of metabolism. Although SCADD does not seem to meet newborn screening criteria, a committee of the National Academy of Clinical Biochemistry could not reach consensus on recommending against adoption of SCADD in newborn screening programs. Therefore SCADD is still included in newborn screening programs in most US states. 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 2 metabolites are both elevated in SCADD, although to different extents.

The diagnosis is confirmed by DNA analysis of the gene that encodes SCAD (ACADS). Currently, 38 different mutations have been reported in patients with SCADD. In addition, 2 common ACADS variants, c.511C>T and c.625G>A, have been found in the general population with prevalence of homozygosity of approximately 0.3 and 5.5%, respectively. Most patients with SCADD are homozygous or compound heterozygous for 1 or 2 of the common ACADS variants or for a combination of one of these variants with an ACADS mutation. Both gene variants may play a modifying role in the pathogenesis of clinical SCADD, by conferring susceptibility to clinical disease.

The SCAD enzyme is a flavoprotein consisting of 4 subunits, each of which contains one molecule of its co-factor 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. FAD binding is important not only for the catalytic activity of flavoproteins but also for their folding, assembly, and/or stability. Because all of the studied ACADS mutations and variants result in protein misfolding, riboflavin therapy might be particularly efficacious in SCADD patients if it can stabilize the affected protein. In addition, riboflavin deficiency is a relatively common condition 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 but rare in Western societies.
Results of riboflavin treatment have previously been reported in only 3 patients with SCADD.\textsuperscript{12,13,30} In 2 of them, riboflavin treatment seemed to be beneficial.\textsuperscript{12,30} In one of them, clinical improvement persisted after cessation of therapy.\textsuperscript{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 SCADD patients and to compare effects between the different \textit{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 \textit{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.\textsuperscript{5} Patients were classified into 3 different genotype groups: 1) \textit{ACADS} mutations on both alleles (mutation/mutation [mut/mut] group; \textit{n}=3); 2) an \textit{ACADS} mutation on one allele and an \textit{ACADS} variant on the other allele or both alleles (mutation/variant [mut/var] group; \textit{n}=8); and 3) \textit{ACADS} variants on both alleles (variant/variant [var/var] group; \textit{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 3 doses with a maximum of 150 mg/day, and ingested during meals. Before (day 0) and 5 weeks after starting treatment (day 35) blood was obtained for C4-C and FAD analyses, and urine was obtained for determination of FAD excretion. During the 7 days preceding day 0 and day 35, 5 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 high-performance liquid chromatography.\textsuperscript{31} Acylcarnitine profiles were determined using electrospray tandem mass spectrometry.\textsuperscript{32} Urine samples were stored at -20°C until analysis and were analyzed for organic acids by gas chromatography/mass spectrometry.\textsuperscript{33}
Clinical signs and symptoms

Both before and 5 weeks after the start of riboflavin therapy, a medical history was taken. At the 5 weeks 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 3 different genotype groups and the Mann-Whitney test to compare 2 different groups. The Wilcoxon signed rank test was used to compare baseline values to 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 1 patient (patient 2, who received high caloric tube feeding, Table 1). A significant difference in blood FAD concentrations was observed between the 3 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 3 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 5 of the 8 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 4 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 3 of these 5 biochemically responding patients, blood FAD levels were measured before and during riboflavin treatment. The increase in FAD levels was significantly higher as compared to 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 4 of the patients (patients 4, 7, 8, and 10), all responding biochemically to riboflavin, a slight clinical improvement was reported 5 weeks after initiation of riboflavin
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 weeks of riboflavin treatment in 16 Dutch SCADD patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Blood FAD (µmol/L)*</th>
<th>Urine FAD (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 35</td>
</tr>
<tr>
<td><strong>Mutation/Mutation group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M/4</td>
<td>c.1138C&gt;T Allele 1</td>
<td>c.1138C&gt;T Allele 2</td>
</tr>
<tr>
<td>2† M/2</td>
<td>c.988C&gt;T, c.625G&gt;A</td>
<td>c.1147C&gt;T</td>
</tr>
<tr>
<td>3 F/7</td>
<td>c.988C&gt;T, c.625G&gt;A</td>
<td>c.1147C&gt;T</td>
</tr>
<tr>
<td><strong>Median values</strong></td>
<td>0.34§</td>
<td>0.35_</td>
</tr>
<tr>
<td><strong>Mutation/Variant group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 M/12</td>
<td>C136C&gt;T, c.625G&gt;A</td>
<td>0.22</td>
</tr>
<tr>
<td>5 F/4</td>
<td>c.1058C&gt;T</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td>6 M/3</td>
<td>c.1058C&gt;T</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td>7 F/6</td>
<td>c.1058C&gt;T</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td>8 M/1</td>
<td>c.1058C&gt;T</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td>9 F/3</td>
<td>c.1058C&gt;T</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td>10 F/14</td>
<td>c.IVS1-6C&gt;A</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td>11 M/7</td>
<td>c.449C&gt;T</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td><strong>Median values</strong></td>
<td>0.27§</td>
<td>0.32_</td>
</tr>
<tr>
<td><strong>Variant/Variant group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 M/2</td>
<td>c.625G&gt;A</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td>13 M/3</td>
<td>c.625G&gt;A</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td>14 F/4</td>
<td>c.625G&gt;A</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td>15 M/17</td>
<td>c.625G&gt;A</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td>16 F/1</td>
<td>c.625G&gt;A</td>
<td>c.625G&gt;A</td>
</tr>
<tr>
<td><strong>Median values</strong></td>
<td>0.28§</td>
<td>0.31_</td>
</tr>
<tr>
<td><strong>Median values of the entire cohort</strong></td>
<td>0.28</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Data of Riboflavin responsive patients are in bold type; NA, not analyzed. *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 5 early morning voids collected within the preceding 7 days. †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 to the other, non-responding, SCADD patients (0.07 µmol/L and 0.02 µmol/L, respectively, P = 0.04).
<table>
<thead>
<tr>
<th>Patient</th>
<th>Blood FAD (µmol/L)*</th>
<th>Urine FAD (mg/L)</th>
<th>Median EMA (µmol/mmol creatinine) in urine**</th>
<th>C4-C (µmol/L) in plasma†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>35</td>
<td>p</td>
<td>Day 0</td>
</tr>
<tr>
<td>1</td>
<td>M/4 c.1138C&gt;T c.1138C&gt;T</td>
<td>0.34</td>
<td>0.31</td>
<td>2.75</td>
</tr>
<tr>
<td>2‡</td>
<td>M/2 c.988C&gt;T, c.625G&gt;A</td>
<td>0.41</td>
<td>0.37</td>
<td>7</td>
</tr>
<tr>
<td>3 F/7 c.988C&gt;T, c.625G&gt;A</td>
<td>0.34</td>
<td>0.35</td>
<td>1.7</td>
<td>6.7</td>
</tr>
<tr>
<td>4 M/12 C136C&gt;T, c.625G&gt;A</td>
<td>0.22</td>
<td>0.35</td>
<td>35</td>
<td>34</td>
</tr>
<tr>
<td>5 F/4 c.1058C&gt;T c.625G&gt;A</td>
<td>0.27</td>
<td>0.30</td>
<td>1.39</td>
<td>7.00</td>
</tr>
<tr>
<td>6 M/3 c.1058C&gt;T c.625G&gt;A</td>
<td>NA</td>
<td>0.34</td>
<td>NA</td>
<td>31.00</td>
</tr>
<tr>
<td>7 F/6 c.1058C&gt;T c.625G&gt;A</td>
<td>0.28</td>
<td>0.35</td>
<td>0.32</td>
<td>14</td>
</tr>
<tr>
<td>8 M/1 c.1058C&gt;T c.625G&gt;A</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>9 F/3 c.1058C&gt;T c.625G&gt;A</td>
<td>0.27</td>
<td>0.26</td>
<td>1.00</td>
<td>5.30</td>
</tr>
<tr>
<td>10 F/14 c.IVS1-6C&gt;A c.625G&gt;A</td>
<td>0.22</td>
<td>0.29</td>
<td>0.80</td>
<td>9.50</td>
</tr>
<tr>
<td>11 M/7 c.449C&gt;T c.625G&gt;A</td>
<td>0.29</td>
<td>0.32</td>
<td>0.52</td>
<td>1.6</td>
</tr>
<tr>
<td>12 M/2 c.625G&gt;A c.625G&gt;A</td>
<td>0.28</td>
<td>0.30</td>
<td>0.79</td>
<td>25.33</td>
</tr>
<tr>
<td>13 M/3 c.625G&gt;A c.625G&gt;A</td>
<td>0.33</td>
<td>0.31</td>
<td>2.30</td>
<td>6.81</td>
</tr>
<tr>
<td>14 F/4 c.625G&gt;A c.625G&gt;A</td>
<td>0.22</td>
<td>0.29</td>
<td>1.92</td>
<td>3.80</td>
</tr>
<tr>
<td>15 M/17 c.625G&gt;A c.625G&gt;A</td>
<td>0.28</td>
<td>0.37</td>
<td>0.29</td>
<td>NA</td>
</tr>
<tr>
<td>16 F/1 c.625G&gt;A c.625G&gt;A</td>
<td>0.26</td>
<td>0.31</td>
<td>0.39</td>
<td>4.5</td>
</tr>
<tr>
<td>Median values</td>
<td>0.27§</td>
<td>0.32</td>
<td>0.9 _</td>
<td>9.5_</td>
</tr>
<tr>
<td>Median values of the entire cohort</td>
<td>0.28 0.31</td>
<td>0.75 _</td>
<td>5.66_</td>
<td></td>
</tr>
</tbody>
</table>

Data of Riboflavin responsive patients are in bold type; NA, not analyzed. *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 5 early morning voids collected within the preceding 7 days. †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 to the other, non-responding, SCADD patients (0.07 μmol/L and 0.02 μmol/L, respectively, P = 0.04).

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/kg/day, with a maximum of 150 mg/day) 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.\textsuperscript{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

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

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

Data of riboflavin-responsive patients (see Table 1) are in bold type.
blood FAD compared with the biochemically non-responding 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, 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 ACADS 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 SCADD individuals identified by newborn screening in future studies.

Four of the 5 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 years.

None of the patients who were homozygous for the c.625G>A ACADS 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 (Tabel 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, focussing
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.
Acknowledgments

We thank the patients with SCADD and their parents for their participation in this study; Thessa Westphal and Klaske Honig for their excellent assistance during all the performed tests; and J.H. van der Lee of the Department of Pediatric Clinical Epidemiology for assistance on statistical analyses.
References


Chapter 6

Exercise testing in patients with short-chain acyl-CoA dehydrogenase deficiency: biochemical responses and effects of riboflavin therapy

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¹Department of Pediatrics, ²Laboratory Genetic Metabolic Diseases, Academic Medical Center, University of Amsterdam, Amsterdam, ³Department of Pediatrics, University Medical Center Utrecht, Utrecht, The Netherlands.
Abstract

Short-chain acyl-CoA dehydrogenase deficiency (SCADD) is a mitochondrial fatty acid oxidation disorder, most frequently associated with neurologic symptoms and/or hypoglycemia. SCADD is biochemically characterized by increased C4-carnitine (C4-C) in blood and ethylmalonic acid (EMA) in urine and caused by rare mutations and/or common gene variants in the SCAD-encoding gene. Previous studies showed that EMA increases and C4-C remains stable during fasting. In addition, EMA and C4-C decreased in response to riboflavin therapy in a subgroup of SCADD patients with a mutation/variant genotype.

Our aim was to examine the biochemical response to exercise in SCADD patients and to assess whether high-dose riboflavin therapy exerts any effects on the observed response. Exercise tests were performed in 3 SCADD patients, all with mutation/variant genotypes and symptoms of exercise intolerance or fatigue. One patient, who responded clinically to riboflavin therapy, was retested during, and 1, and 2 years after riboflavin therapy.

C4-C concentrations in plasma increased in response to exercise, while EMA excretion remained stable. In the patient who was retested during riboflavin therapy, no exercise induced increase of plasma C4-C was seen. However, this C4-C increase was present again 2 years after cessation of riboflavin therapy, though she was still without clinical symptoms. As C4-C increases in SCADD patients during exercise and EMA increases during fasting, preferred tissue-specific pathways might exist. In addition, high-dose riboflavin therapy may prevent C4-C increase during exercise, but appears not to be related to any clinical effect in this particular patient.
Introduction

Short-chain acyl-CoA dehydrogenase (SCAD, EC 1.3.99.2) deficiency (SCADD, OMIM 201470) is an autosomal recessive inborn error of mitochondrial fatty acid \( \beta \)-oxidation. SCADD is relatively common,\(^1\) and is most frequently diagnosed as a result of investigations for developmental delay, epilepsy, behavioral disorders, hypoglycemia and hypotonia.\(^{1-13}\) Although SCADD appears to go without any clinical significance in many individuals,\(^1;12\) SCADD is included in newborn screening programs in most US states.\(^14\) In order to obtain insight into the pathophysiological consequences of SCADD, we previously studied the biochemical effects of fasting and fat-loading in SCADD patients.\(^15\) In order to study the efficacy of potential treatments, we assessed the effects of high-dose riboflavin therapy on the biochemical SCADD characteristics and clinical status in SCADD patients.\(^16\) Studies on the biochemical effects of exercise and the effect of riboflavin on the biochemical effects during exercise in SCADD patients have not yet been performed.

The SCAD enzyme catalyzes the dehydrogenation of butyryl-CoA (C\(_4\)-CoA) and is the first enzyme involved in the short-chain fatty acid \( \beta \)-oxidation spiral. When SCAD activity is impaired, its substrate C\(_4\)-CoA accumulates and is subsequently converted into different metabolites, including the corresponding carnitine-ester (butyrylcarnitine, C\(_4\)-C), butyrate, and ethylmalonic acid (EMA) (Figure 1). C\(_4\)-C can be measured in blood and EMA can be measured in urine. In our previous study we demonstrated that EMA increased significantly during fasting, while C\(_4\)-C remained stable.\(^15\)

The SCAD enzyme is a flavoprotein consisting of 4 subunits, each of which contains one molecule of its co-factor flavin adenine dinucleotide (FAD). Riboflavin, vitamin B\(_2\) (7,8-dimethyl-10-ribityl-isoalloxazine) is the precursor of FAD and is predominantly

![Figure 1. Metabolic Fate of Butyryl-CoA in SCADD](image-url)
ingested through the consumption of milk and dairy products. FAD binding is important not only for the catalytic activity of flavoproteins, but also for folding, assembly, and/or stability. Treatment with riboflavin could therefore be a potentially effective therapy in SCADD. Indeed, we previously demonstrated a biochemical effect of high-dose riboflavin supplementation in a subgroup of SCADD patients.

The diagnosis of SCADD is based on the presence of inactivating mutations and/or common variants of the SCAD-encoding gene (ACADS). The majority of SCADD patients are homozygous or compound heterozygous for either 1 or 2 common ACADS variants or for ACADS variants in combination with an ACADS mutation. The ACADS variants have been found in the general population with a remarkably high prevalence of homozygosity, with frequencies of approximately 0.3% for the c.511C>T and 5.5% for the c.625G>A variant. These variants are thought to play modifying roles in the pathogenesis of SCADD by conferring susceptibility for clinical disease. Those SCADD patients showing at least biochemical benefit from riboflavin therapy all carry a mutation on one ACADS allele and a variant (c.625G>A) on the other. This points to a potential role of the c.625G>A variant leading to functional SCADD caused by decreased FAD affinity and/or SCAD protein instability.

The mitochondrial oxidation of fatty acids plays an important role in energy production, not only during periods of prolonged fasting, but also during moderately intense exercise. Different pathophysiological mechanisms that underlie the inborn errors of fatty acid oxidation (FAO) can be distinguished including the following: 1) inadequate supply of energy, 2) sequestration or loss of vital components of intermediary metabolism, and 3) accumulation of toxic metabolites. The results of our previous study, showing EMA increase during fasting as the only biochemical abnormality in SCADD patients, led us to suspect that, with respect to SCADD, the latter mechanism is the most likely one.

The aims of the current study were: a) to examine the effects of moderately intense exercise on biochemical profiles in SCADD patients and b) to assess whether high-dose riboflavin therapy exerts any effects on these profiles during exercise.

Methods

Patients

Three patients, 1 male and 2 females, aged 6, 8, and 13 years respectively, who all suffered from exercise intolerance or fatigue, were included in the study group. All patients were diagnosed with SCADD on the basis of increased C4-C in plasma and/or increased EMA in urine under non-stressed conditions on at least two occasions, and the presence of a mutation and/or the c.511C>T or c.625G>A variants on each ACADS allele. Genetic changes indicating SCADD were established by sequence analysis of all exons and flanking intronic sequences. The genotypes and clinical phenotypes of the
participating patients are shown in the table. The selected patients were part of the Dutch SCADD cohort described previously.1

Tests
All tests were performed between January 2002 and January 2008. Written informed consent was obtained from the parents and/or legal representatives of all patients participating in this study. The study was reviewed and approved by the Medical Ethics Committee of the Academic Medical Center.

Exercise protocol
The exercise test consisted of 60 minutes of cycling on a magnetic braked cycle ergometer (E5R, Tunturi Oy Ltd, Finland). Heart rates were monitored continuously and each patient exercised at 60% of his or her predicted maximum heart rate. The value for maximum heart rate was calculated as follows: 60% of maximum heart rate (beats per minute) = (208 – 0.7 x age) x 0.6.28 Blood samples were collected immediately prior to cycling (at t=0), during cycling (at t = 15, 30, 45, and 60 minutes), and in the case of patient 3, 1 hour after the exercise (at t = 120 minutes). Urine samples were collected prior to and after the exercise.

Riboflavin treatment and assessment in patient 3
Riboflavin was administered to patient 3 in a total dose of 150 mg per day divided in 3 doses, ingested during meals. This patient was studied on 4 separate occasions. A first exercise test was performed because of fatigue. As she appeared to respond clinically to riboflavin therapy, a second exercise test was performed after 4 months of therapy and a third and fourth test after riboflavin therapy had been stopped for 1 and 2 years respectively.

Blood and urine analysis
Blood samples were analyzed for glucose, nonesterified fatty acids (NEFA), creatine kinase (CK), alanine-aminotransferase (ALAT), and aspartate-aminotransferase (ASAT). In addition biochemical analyses of blood samples were performed to explore lactate,

<table>
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<tr>
<th>Pt</th>
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<th>Genotype†</th>
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<tr>
<td>1</td>
<td>6 m</td>
<td>c.1058C&gt;T</td>
<td>c.625G&gt;A</td>
<td>Hypoglycemia, fatigue</td>
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<td>2</td>
<td>8 f</td>
<td>c.1058C&gt;T</td>
<td>c.625G&gt;A</td>
<td>Mild developmental delay, exercise intolerance</td>
</tr>
<tr>
<td>3</td>
<td>13 f</td>
<td>c.IVS1-6C&gt;A</td>
<td>c.625G&gt;A</td>
<td>Fatigue</td>
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Abbreviations: Pt, patient; y, years; G, gender; h, hours. *Age when tested. †Gene variants in regular type, mutations in bold type.
pyruvate, ketone bodies (KB), and acylcarnitine profiles. Acylcarnitine profiles were determined using electrospray tandem mass spectrometry. Blood samples for lactate, pyruvate, and KB were immediately deproteinized with perchloric acid and stored on ice, followed by quantitative determination of metabolites using standard spectrophotometric or fluorimetric methods. All samples were delivered to the laboratory within 10 minutes of collection. Urine samples were analyzed for organic acids by gas chromatography/mass spectrometry, and stored at -20°C until analysis.

Interpretation of test results
EMA was considered to be increased if patients had >15 μmol/mmol creatinine for children younger than 2 years and >8 μmol/mmol creatinine for children aged 2 years and older. The upper reference range for C4-C was 0.58 μmol/L.

Results
Exercise tests
None of the patients showed any clinical signs of symptoms during or after the 60 minutes of exercise and remained asymptomatic during and after the test. Glucose, CK, FFA, ALAT, and ASAT remained normal and stable during and after the test. Free carnitine levels remained stable in patients 2 and 3 (data not shown, not measured in patient 1). C4-C values showed a clear increase in patients 2 and 3 (Figure 2A, not measured in patient 1). The variation in urinary EMA excretion was comparable to the at random EMA fluctuations reported previously (Figure 2B).

Exercise test results in response to riboflavin therapy in patient 3
On riboflavin therapy, C4-C levels during exercise decreased compared to baseline levels (Figure 3). One year after stopping riboflavin treatment, C4-C levels had increased again but were still lower when compared to the levels of the baseline test (Figure 3). After 2 years without riboflavin therapy, and still without any clinical signs or symptoms, a clear C4-C increase in response to exercise was again detected (Figure 3). EMA excretion was similar during the 3 consecutive tests (data not shown).

Discussion
The present study is the first to describe the biochemical response to exercise and the effects of riboflavin treatment on this response in SCADD patients. In our previous study, an increase in EMA excretion was observed during fasting, while C4-C remained stable. The current study demonstrates the opposite: C4-C increases during exercise in both
patients tested, while EMA excretion remained stable. The increase in EMA excretion, as observed during fasting is likely due to the accumulation of C4-CoA, the substrate of SCAD, which is converted into ethylmalonyl-CoA by propionyl-CoA carboxylase and subsequently into EMA by one of the mitochondrial acyl-CoA hydrolases. The increase in C4-C, as observed during exercise, is most likely caused by the increased production of C4-C from C4-CoA by the mitochondrial enzyme carnitine acetyltransferase. Even though our study only included a limited number of patients, the observed profiles might suggest the existence of a preferred pathway towards EMA during fasting and towards C4-C during exercise. This might be based on higher intramitochondrial carnitine concentrations in muscle compared to liver, resulting in a higher proportion of C4-CoA being converted into C4-C.31

Figure 2. Biochemical characteristics of short-chain acyl-CoA dehydrogenase deficiency (SCADD) during exercise in SCADD Patients. The profiles of C4-Carnitine (C4-C) in 2 SCADD patients and ethylmalonic acid (EMA) in 3 SCADD patients during exercise are shown. A, C4-C. The dashed line represents the upper limit of normal levels (0.58 μmol/L). B, EMA. The dashed line represents the upper limit of normal levels (8 μmol/mmol creatinine).

Figure 3. C4-Carnitine (C4-C) during exercise before, during, and 1, and 2 years after riboflavin therapy in patient 3.
No studies on the toxicity of C4-C have been performed. As discussed previously the accumulation of potentially toxic metabolites is the most likely mechanism to be involved in the pathophysiology of SCADD.\textsuperscript{1} However, previous studies showed that patients with the highest levels of EMA and C4-C (the mutation/mutation genotype group) were not more severely affected with respect to ketogenesis and clinical symptoms when compared with patients with lower EMA and C4-C levels (the mutation/variant or variant/variant genotype groups).\textsuperscript{1,5,15} Therefore, no conclusions can yet be made regarding a potential toxic effect from C4-C and/or other metabolites derived from butyryl-CoA.

A clear biochemical response to riboflavin therapy was demonstrated by the prevention of increased C4-C values during exercise in patient 3. As exercise stimulates FAO, repeating these tests during riboflavin treatment offered the potential to assess its functional efficacy in SCADD patients. The observation that C4-C levels in response to exercise clearly increased again 2 years after cessation of riboflavin therapy but without any changes in clinical signs and symptoms, points to 2 different aspects. First of all, discontinuation of riboflavin therapy only showed its effects 2 years later. This can be explained by the fact that there is only little destruction of riboflavin associated with its function in metabolism,\textsuperscript{32} together with the observation that riboflavin intake was high enough to provide for normal FAD levels preceding riboflavin therapy in this patient. Secondly, it supports the hypothesis that the observed responses of SCADD patients to riboflavin are of a biochemical nature and are not related to any improvement in clinical disease.\textsuperscript{16}

In summary, we have demonstrated that C4-C increases during exercise in SCADD patients, whereas during fasting the urinary excretion of EMA increased. We hypothesize that preferred pathways might exist towards EMA during fasting and towards C4-C during exercise. This could be due to a higher intramitochondrial carnitine concentration in muscle as compared to liver. In addition we showed that high-dose riboflavin therapy fully prevented C4-C increase during exercise. However, as the C4-C increase during exercise was present again 2 years after cessation of therapy but was not accompanied by any deterioration in clinical symptoms, the biochemical effect of riboflavin does not seem to be related to any clinical effect in the patient tested.

Acknowledgments
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Chapter 7

Clinical aspects of short-chain acyl-CoA dehydrogenase deficiency

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Abstract

Short-chain acyl-CoA dehydrogenase deficiency (SCADD) is an autosomal recessive inborn error of mitochondrial fatty acid oxidation. SCADD is biochemically characterized by increased C4-carnitine in plasma and ethylmalonic acid in urine. The diagnosis of SCADD is confirmed by DNA analysis showing SCAD gene mutations and/or variants. SCAD gene variants are present in homozygous form in approximately 6% of the general population and considered to confer susceptibility to development of clinical disease.

Clinically, SCADD generally appears to present early in life and to be most frequently associated with developmental delay, hypotonia, epilepsy, behavioral disorders, and hypoglycemia. However, these symptoms often ameliorate and even disappear spontaneously during follow-up and were found to be unrelated to the SCAD genotype. In addition, in some cases, symptoms initially attributed to SCADD could later be explained by other causes. Finally, SCADD relatives of SCADD patients as well as almost all SCADD individuals diagnosed by neonatal screening, remained asymptomatic during follow-up.

This potential lack of clinical consequences of SCADD has several implications. First, the diagnosis SCADD should never preclude extension of the diagnostic workup for other potential causes of the observed symptoms. Second, patients and parents should be clearly informed about the potential lack of relevance of the disorder, in order to avoid unfounded anxiety. Furthermore, to date, SCADD is not an optimal candidate for inclusion in newborn screening programs. More studies are needed to fully establish the relevance of SCADD and solve the question whether SCADD is involved in a multifactorial disease or represents a non-disease.
Introduction

Short-chain acyl-CoA dehydrogenase (SCAD, EC 1.3.99.2) deficiency (SCADD, OMIM 201470) is an autosomal recessive inborn error of mitochondrial fatty acid oxidation (FAO). SCAD catalyzes the dehydrogenation of butyryl-CoA (C4-CoA) during the first step of the short-chain fatty acid β-oxidation spiral. Impaired SCAD activity results in accumulation of its substrate (C4-CoA) and the subsequent production of alternative metabolites including the following: 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, measured in blood and EMA, measured in urine, are generally used as biochemical markers for SCADD.

The diagnosis of SCADD is usually confirmed by DNA analysis. The majority of SCADD patients are homozygous or compound heterozygous for 2 common variants of the SCAD-encoding gene (ACADS), or for ACADS variants in combination with an inactivating mutation.\textsuperscript{1-4} Up to 70 different inactivating ACADS mutations have been reported so far.\textsuperscript{1-3,5-12} There is a remarkably high prevalence of homozygosity for ACADS variants in the general population, with frequencies of approximately 0.3% for the p.R171W (c.511C>T) and 5.5% for the p.G209S (c.625G>A) variant.\textsuperscript{13,14} Homozygosity for these variants is considered to confer susceptibility to clinical disease.\textsuperscript{7,8,15}

Most SCADD patients have been diagnosed as a result of investigations for neurological symptoms and/or hypoglycemia.\textsuperscript{1-4} There is, however, debate on the clinical relevance of SCADD.\textsuperscript{3,16} Nevertheless, newborns are screened for SCADD in the USA.\textsuperscript{17} In this manuscript, we will summarize the clinical aspects of SCADD and discuss the clinical relevance of this inborn error of metabolism.

Clinical symptoms in SCADD

Symptoms in clinically identified patients

The first SCADD patient was originally reported by Amendt and co-workers\textsuperscript{18} and subsequently genetically confirmed by Naito in 1990.\textsuperscript{5} The patient was reported to suffer from lethargy, hypertonia, and circulatory problems with metabolic acidosis during her first week of life. Although she was reported to show normal growth and development and without recurrence of metabolic acidosis up to the age of 2 years, Bhala \textit{et al.} later reported that this patient had died, without reporting clinical details.\textsuperscript{19} As this publication included another SCADD patient who died, after initial presentation with severe skeletal muscle hypotonia, a devastating clinical course of SCADD was suggested. However, the same publication included another 2 cases, initially presenting with “possible hyperactivity” and “probable seizure activity”, but with a normal follow-up. Initially, a few other patients with SCADD had been reported,\textsuperscript{18-20} however in these patients the
diagnosis was, to our knowledge, not genetically confirmed. As the definitive diagnosis of SCADD requires molecular testing, these patients are excluded from this review.

Subsequently a large cohort of SCADD patients was presented by Corydon et al. and several case reports were published. Based on these publications, SCADD appeared to be associated with a wide spectrum of clinical signs and symptoms, including developmental delay, hypotonia, epilepsy, and hypoglycemia, and in solitary cases dysmorphic features, vomiting, failure to thrive, hepatic dysfunction after premature delivery, and bilateral optic atrophy. One case report suggested the association between SCADD and acute fatty liver of pregnancy in the mother. Again a striking spectrum was observed in patient outcome. Outcome was reported for 7 patients, of whom 5 fully recovered, 1 slowly progressed, and 1 died.

In 2006, we reported data on 31 Dutch SCADD patients. The most frequently reported symptom in this cohort was developmental delay, followed by epilepsy, behavioral disorders, and hypoglycemia. Behavioral disorders, observed in 8 out of the 31 patients, had not been previously reported in SCADD patients. Remarkably, most of the clinically severely affected patients belonged to the group of patients homozygous for the c.625G>A variant. In 4 patients, additional diagnoses, that were highly likely causing the clinical symptoms, were made after the initial diagnosis of SCADD.

In 2008, Tein and co-workers published a study on 10 SCADD patients. Developmental delay was again the most common symptom, but this time hypotonia was also as frequent. In addition this study reported for the first time a relatively high prevalence of lethargy (5 patients), myopathy (4 patients), and facial weakness (3 patients). All patients in this study were of Ashkenazi Jewish descent, carried the c.319C>T mutation and were either homozygous for this mutation or had the c.625G>A variant on the other allele. In 2 patients with myopathy, a muscle biopsy revealed multiminicore disease, a rare congenital myopathic disorder. However, other genetic causes for multiminicore disease, in particular mutations in SEPN1 and RYR1, which are present in about 50% of cases, had not been excluded.

In the same year, Pedersen and co-workers published a study on a very large cohort of 114 SCADD patients from Europe, New Zealand and Canada. Again developmental delay was the most frequently reported clinical sign, in combination with hypotonia, seizures, and failure to thrive. In addition, several patients were reported to have failure to thrive and hypotonia without developmental delay and a smaller group had dysmorphic features.

A third study, also published this same year, by Waisbren and co-workers, reported on another 6 clinically identified patients. In 3 of them newborn screening had failed to detect SCADD and in the other 3 no screening for SCADD was performed. The most significant symptoms in these 6 patients were developmental delay, hypotonia, feeding problems, and failure to thrive and epilepsy.
Age of presentation, symptom transience and genotype-phenotype relation in clinically identified patients

It appears that clinical signs and symptoms in SCADD patients generally present early in life, with almost all patients presenting under the age of 5 years. Symptoms were transient in 9 out of the 31 patients in the van Maldegem study, and 2 out of 6 patients in the Waisbren study. Furthermore, no association could be made between genotype and clinical phenotype in the patients from the Pedersen and the van Maldegem study.

Clinical symptoms in SCADD individuals identified by newborns screening

With the implementation of newborn screening for SCADD in the U.S.A and Australia, the clinical spectrum of SCADD has expanded. Several follow-up studies of SCADD newborns diagnosed through newborn screening were published within the last few years. The first one reported 17 SCADD newborns who all remained symptom-free at follow-up during their first 2 years of life. Of the 3 patients reported by Koeberl et al., 1 developed seizures and a cerebral infarction at the age of 10 weeks, while the other 2 patients remained symptom-free during their first 3 years of life. One out of the 8 children reported by Waisbren and co-workers did show developmental delay consisting of a language delay at the age of 2 years. Jethva and Ficicioglu reported a group of 14 children with SCADD of whom 11 were identified by newborn screening and 3 diagnosed by screening of sibs diagnosed through newborn screening. During a follow-up of 1 to 7 years, in 2 of them (siblings) speech delay was diagnosed. A causative relation between this speech delay and SCADD was considered unlikely, as both parents had learning disabilities, suggesting other causes. All 4 Australian SCADD children diagnosed by newborn screening and studied during 6 years remained symptom-free. SCADD has meanwhile been excluded from the Australian screening panel because of supposed lack of clinical significance.

Figure. Comparison of the number of symptoms in metabolically screened patients to the number of symptoms at presentation in the Dutch short-chain acyl-CoA dehydrogenase (SCADD) patients group
An interesting finding by Waisbren and co-workers is a relatively high prevalence of pregnancy complications in the mothers of children diagnosed with SCADD by newborn screening as well as in mothers of the group of SCADD patients diagnosed because of clinical symptoms. In 5 out of 14 patients, hypertension, maternal bradycardia, pre-eclampsia, and mild HELLP syndrome were reported in the mothers. This suggests that fetal SCADD, like other FAO disorders such as LCHAD deficiency, might be associated with pregnancy complications as previously suggested. However, in almost 30% of all pregnancies in the Dutch population mild to moderate pregnancy complications are reported, including hypertensive disorders and pre-eclampsia. Furthermore, the association between SCADD and maternal pregnancy complications was not detected in the Dutch cohort of SCADD patients as maternal disease during pregnancy was only reported in 4 out of 24 patients (17%) on whom pregnancy details were known (unpublished data).

Family studies
Thirty-seven relatives (20 parents and 17 sibs) of the SCADD patients from the Dutch cohort were investigated for their ACADS genotype and 9 of them were found to have the same ACADS genotype as the proband. Except for 1 father with an ACADS genotype homozygous for the c.625G>A variant, all relatives with an ACADS genotype identical to the proband were found to have increased C4-C and/or EMA. Eight relatives had always been healthy, while 1 had a history of transient food refusal during her first year of life.

Clinical significance
The clinical studies discussed above show that symptoms in SCADD generally present early in life and most frequently concern developmental delay, hypotonia, epilepsy, behavioral disorders, and hypoglycemia. Signs and symptoms often ameliorate and disappear completely during follow-up. In addition, they can sometimes be explained by other causes and are not related to the ACADS genotype. Finally, almost all relatives diagnosed with SCADD, as well as almost all individuals detected by newborn screening, remain fully asymptomatic. Based on these observations, one may question the clinical relevance of SCADD. Indeed, we hypothesize that the association between the reported signs and symptoms and the diagnosis of mutations or gene variants in the ACADS gene could be coincidental.

As a first step to study this, we searched the database of the laboratory for metabolic diseases in our centre for the most frequently reported symptoms of patients for whom metabolic studies were requested between November 2007 and November 2008. Developmental delay was by far the most frequently reported, followed by behavioral disorders, epilepsy, hypotonia, and hypoglycemia. The incidence of these symptoms is comparable to that in the SCADD patients group (Figure), suggesting at least that there is no specific cluster of clinical signs and symptoms in SCADD, and that the association might indeed be coincidental.
Implications with respect to policy towards SCADD patients and families

The probable lack of clinical significance of SCADD has important implications for the clinical management and counselling of SCADD patients and families. First, the diagnosis of SCADD should never preclude a full diagnostic workup for other potential causes of the symptoms. Any delay in doing so might postpone the diagnosis of other potentially treatable causes. Second, patients and parents as well as their physicians should be clearly informed about the potential lack of clinical relevance of the detected biochemical and genetic abnormalities concerning SCADD, once the diagnosis of SCADD has been made. Furthermore, without a clear clinical phenotype of SCADD to date, SCADD is not an optimal candidate for inclusion in newborn screening programs. Meanwhile, further studies are needed to fully unravel the implications of SCADD in order to answer the question whether SCADD is involved in a multifactorial disease or represents a non-disease.
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High prevalence of short-chain acyl-CoA dehydrogenase deficiency in the Netherlands, but no association with epilepsy of unknown origin in childhood

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Submitted
Abstract

Short-chain acyl-CoA dehydrogenase deficiency (SCADD) is an autosomal recessive inborn error of metabolism, most frequently associated with developmental delay and/or epilepsy. Most SCADD patients carry common SCAD-encoding gene (ACADS) variants or ACADS variants in combination with a mutation. The ACADS variants are considered to confer susceptibility to development of clinical disease. The most common ACADS mutation in the Netherlands is the c.1058C>T. C4-Carnitine, one of the biochemical SCADD characteristics is increased in almost all patients. Epilepsy is a frequent finding in childhood and often remains unexplained. However, studies for inborn errors of metabolism such as SCADD are often not performed. Because the prevalence of SCADD is much higher than the number of patients detected, patients with epilepsy related to SCADD may remain undiagnosed.

To test this hypothesis and to further estimate the extent of the Dutch SCADD population, we performed a case-referent study in 131 paediatric patients with epilepsy and 909 anonymous newborns. To investigate the presence of the 2 common ACADS variants and the c.1058C>T mutation and to measure C4-Carnitine, DNA PCR-RFLP analysis and electrospray tandem mass spectrometry were performed on blood spot samples.

Overall, the 2 ACADS variants and c.1058C>T mutation were detected in either homozygous or compound heterozygous forms in 9.2% of the epilepsy and 7.5% of the reference group. In none of the epilepsy patients C4-Carnitine was increased. This implies that the birth prevalence of SCADD with a mutation/variant genotype in the Netherlands is > 1:1000, but does not support an association between SCADD and epilepsy. Therefore, SCADD studies cannot be recommended in epilepsy of unknown origin in childhood.
Introduction

Short-chain acyl-CoA dehydrogenase (SCAD) is the first enzyme of the short-chain β-oxidation spiral catalyzing the dehydrogenation of short-chain fatty acids. SCAD deficiency (SCADD) is a relatively newly recognized inborn error of fatty acid oxidation and has been associated with a remarkable variety of clinical symptoms. SCADD is probably one of the more common inborn errors of metabolism.1

Butyryl-CoA (C4-CoA) is the main substrate for the SCAD enzyme and accumulates in mitochondria of patients with decreased SCAD activity, which subsequently leads to increased levels of butyrylcarnitine (C4-C) and ethylmalonic acid (EMA). EMA can best be measured in urine, whereas C4-C can best be measured in plasma and blood spots dried on filter paper. These 2 metabolites constitute the major biochemical features of SCADD.

Diagnosis of SCADD is usually confirmed by DNA analyses.1 The majority of SCADD patients are homozygotes or compound heterozygotes for two common SCAD-encoding gene (ACADS) variants, or for ACADS variants in combination with an inactivating mutation.1-4 Homozygosity for ACADS variants has been found in the general population with a remarkably high prevalence of approximately 0.3% for the c.511C>T and 5.5% for the c.625G>A variant,5,6 and is considered to confer susceptibility to development of clinical disease.7,9 More than 70 different ACADS mutations have been reported so far.1,3,7,8,10-15 In the majority of Dutch patients one specific mutation, the c.1058C>T, is present next to the c.625G>A variant on the other allele.1 The c.1058C>T mutation is located on the c.625G and c.511C ACADS allele. Overall an ACADS genotype consisting of this specific mutation and/or ACADS variants was present in 61% of the Dutch SCADD patients.1

Most of the reported SCADD patients were initially investigated because of neurological symptoms and/or hypoglycemia.1-4,7 In our previous study on 31 Dutch SCADD patients, developmental delay was reported in 52% and epilepsy in 35% of the patients.1 Behavioral disorders and hypoglycemia were reported in 26% and 19% of this patient group respectively. No correlation between genotype and phenotype could be established and in some patients other diagnoses, explaining the symptoms initially leading to metabolic studies, were identified.

Epilepsy is a frequent finding in childhood. Studies from Europe and North America report rates from 3.6 to 6.5/1000 children.16-21 Diagnostic studies fail to establish the cause of the epilepsy in up to 30% of the patients.22 However, in patients with idiopathic childhood epilepsy, studies for inborn errors of metabolism such as SCADD as a potential cause are often not performed. This, plus the fact that epilepsy is a frequent symptom in SCADD and the observation that the prevalence of SCADD is much higher than the number of patients detected by metabolic studies,1 suggests that patients with epilepsy related to SCADD may well remain undiagnosed.

In order to test this hypothesis we studied a group of pediatric patients with epilepsy for the presence of SCADD. This way we aimed to achieve better knowledge on the
need for SCADD studies in epilepsy patients. In addition we aimed to further estimate the extent of the group of SCADD individuals in the Netherlands by establishing the frequency of the c.511C>T variant and the c.1058C>T mutation in the Dutch population.

Patients and Methods

Patients and material
We performed a case-referent study in 131 pediatric patients with epilepsy and 909 anonymous newborns. Blood spots of the reference group were obtained from the RIVM (National Institute for Public Health and the Environment, the Dutch Institute involved in newborn screening). The sample of newborn screening cards used was drawn proportionally to the number of live births in each of the 14 screening districts, guaranteeing a demographic representation of the Dutch population. As Dutch newborn screening cards are stored for 5 years for reasons of possible need for further testing, the obtained screening cards were consequently >5 years old. The newborn screening cards had been stored at 4°C and were analyzed anonymously.

The study was performed in a pediatric patient group, with patients aged 16 years or younger at the time of participation. As all patients in the Dutch SCADD patients group were < 8 years when epileptic symptoms started, we restricted the epilepsy group to those with a debut of epilepsy < 8 years. Exclusion criteria were a known or suspected cause of the epilepsy and previously performed metabolic studies including SCADD investigations.

Patients with epilepsy were recruited via pediatricians and neurologists within the AMC Amsterdam, and 3 Dutch epilepsy centres. Patient and symptom characteristics were obtained by taking questionnaires answered by the parents or legal representatives.

Written informed consent was obtained from the patients and/or parents/legal representatives of all patients. The study protocol was approved by the Medical Ethics Committee of the Academic Medical Center in Amsterdam and the RIVM.

DNA polymerase chain reaction restriction- fragment length polymorphism analysis
DNA was extracted from bloodspot samples using Chelex 100 (BioRad) essentially as described before, but with the following modifications. Bloodspot samples (6 mm diameter) were washed overnight at 4°C with 1 mL of sterile water. The next day, the supernatant was discarded, 200 μL of Chelex (50 g/L, pH 10.6) was added and the sample was incubated at 56°C for 30 min. Subsequently, the samples were mixed for 10 s, centrifuged (3 min, 10 000 x g), incubated during 8 min at 96°C, mixed again for 10 s, and centrifuged (3 min, 10 000 x g). 14.5μL of supernatant was used in a 25 μL PCR reaction.
The extracted DNA was subjected to polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis to determine the presence of the c.625G>A, and c.511C>T variant, and c.1058C>T mutation in the \textit{ACADS} gene. Concerning the c.625G>A variant, a RFLP assay as described before was used. In order to identify the c.511C>T variant and the c.1058C>T mutation, new PCR-RFLP assays were developed.

The c.511C>T variant creates a \textit{MvaI} restriction site. Exon 5 contains an additional \textit{MvaI} site 42 bp downstream used as an internal control for restriction. The upstream endogenous \textit{MvaI} site was destroyed by a C>A change (bold) in the forward primer in order to increase the size of the restriction product. For the PCR-RFLP analysis, exon 5 of the \textit{ACADS} gene is amplified from 14.5 \(\mu\)L extracted DNA in a 25 \(\mu\)L PCR reaction as described before, except for a 1.25 mmol/L MgCl\(_2\) concentration. The following primers were used: forward 5'-tgtaaaacgacggccagt CGT GCG CTG AGC ACT GGG TCT-3', reverse 5'-caggaaacagctatgacc TCG AAG CCT CCC AGG CAT TGG TGA-3'. Like the reverse primer for the PCR-RFLP analysis of the c.625G>A variant, the forward and reverse primer for determination of the c.511C>T variant have an M13-rev and -21M13 extension (small characters), which can be used for sequence analysis.

The c.1058C>T mutation creates an \textit{Eco130I} (StyI) restriction site. Exon 9 contains an additional \textit{Eco130I} (StyI) site 18 bp upstream used as an internal control for restriction. For this PCR-RFLP analysis, exon 9 of the \textit{ACADS} gene is amplified from 14.5 \(\mu\)L extracted DNA in a 25 \(\mu\)L PCR reaction as described before with 1.0 mmol/L MgCl\(_2\) using 5'-tgtaaaacgacggccagt GGG AAG GCT CTG ACT GTA CC-3' as the forward primer and 5'-caggaaacagctatgacc CAG GAT CTG GAT GGC CTG AG-3' as the reversed primer. These primers also have an M13-rev and -21M13 extension (small characters). For DNA amplification the PTC 100 thermocycler was programmed as follows: Denaturation for 2 minutes at 96\(^\circ\)C, followed by 5 cycles of 30 s at 96\(^\circ\)C, 30 s at 55\(^\circ\)C, and 30 s at 72\(^\circ\)C and subsequently 30 cycles of 30 s at 94\(^\circ\)C, 30 s at 55\(^\circ\)C, and 30 s at 72\(^\circ\)C, with a final step of 15 minutes at 72\(^\circ\)C.

**RFLP c.625G>A.** To 25 \(\mu\)L PCR product 2.9 \(\mu\)L Buffer M and 5U \textit{DdeI} (Roche) were added followed by incubation overnight at 37\(^\circ\)C. The non-digested PCR fragment of the c.625G>A variant comprises 226 bp. The digested PCR product shows 2 restriction fragments of 208 and 18 bp in a non-carrier of the c.625G>A variant and 3 fragments of 180, 28, and 18 bp when the c.625G>A variant is present on both alleles. Consequently, in case of heterozygosity for the c.625G>A variant fragments of 208, 180, 28, and 18 bp will be observed.

**RFLP c.511C>T.** To 25 \(\mu\)L PCR product 2.9 \(\mu\)L Buffer R and 10 U \textit{MvaI} (Fermentas) were added followed by incubation overnight at 37\(^\circ\)C. The non-digested PCR fragment of the c.511C>T variant comprises 174 bp. The digested PCR product shows 2 restriction fragments of 208 and 18 bp in a non-carrier of the c.625G>A variant and 3 fragments of 180, 28, and 18 bp when the c.625G>A variant is present on both alleles. Consequently, in case of heterozygosity for the c.625G>A variant fragments of 208, 180, 28, and 18 bp will be observed.

**RFLP c.1058C>T.** To 25 \(\mu\)L PCR product 2.9 \(\mu\)L Buffer O and 10 U \textit{Eco130I} (StyI) (Fermentas) were added followed by incubation at 37\(^\circ\)C. The non-digested PCR fragment of the
**Figure 1.** PCR-RFLP analysis of the c.511C>T variant

**Upper part,** Schematic representation of the relevant part of the *ACADS* gene. The primers are indicated by *horizontal arrows*; positions of the *Mva*I restriction sites are indicated by *vertical arrows*. **Lower part,** Ethidium bromide-stained agarose gel showing PCR-RFLP analysis using DNA extracted from dried blood spots of reference subjects (1-16) and a heterozygous (c.511C>T/C) control subject (C). Two reference subjects (8, 13) and the control subject show heterozygosity for the c.511C>T variant. PCR products were either directly loaded (·) or digested with *Mva*I before electrophoresis. M=100 bp marker.
Figure 2. PCR-RFLP analysis of the c.1058C>T mutation

Upper part, Schematic representation of the relevant part of ACADS. The primers are indicated by *horizontal arrows*; positions of the Eco130I restriction sites are indicated by *vertical arrows*. Lower part, Ethidium bromide-stained agarose gel showing PCR-RFLP analysis using DNA extracted from dried blood spots of reference subjects (1-16) and a heterozygous (c.1058C>T/C) control subject (C). One reference subject (4) and the control subject show heterozygosity for the c.1058C>T mutation. PCR products were either directly loaded (⊥) or digested with Eco130I before electrophoresis. M=100 bp marker.

c.1058C: CCTCGG
c.1058T: CCTTGG
c.1058C>T mutation comprises 238 bp. The digested PCR product shows 2 fragments of 181 and 57 bp in a non-carrier and 4 fragments of 181, 163, 57, and 18 bp when the c.1058C>T mutation is present on one of the alleles form (Figure 2).

The restriction fragments were analyzed on a 2% (w/v) agarose gel by ethidium bromide staining. Before loading 1/10 volume loading dye (ficoll 400 150g/L, orange G 1.5g/L, and 0.14g/L Xylene Xyanol FF) was added to the samples. As a reference a 100 bp marker (Invitrogen) was used in order to estimate the size of the fragments.

Acylcarnitine analyses

In order to identify individuals with SCADD due to an ACADS genotype not included in the ACADS analysis, C4-C analysis was performed in all blood spots. From all blood spots samples with a diameter of 6 mm, equivalent to 10 µL of blood were punched for determination of C4-carnitine and C0-carnitine levels using electrospray tandem mass spectrometry. The acylcarnitines were extracted with methanol and analyzed as their butyl esters using (2H3)-, (2H3)-C3-, (2H3)-C8-, (2H3)-C16- carnitine as internal standards. The reference range used for the C4-carnitine values was 0.14-0.87 µmol/L.

Statistical analyses

SPSS (SPSS 12.0.1), Graphpad Prism 3.0, and CIA (version 1, 1989) software programs were used for analyzing the data from the DNA and acylcarnitine analyses.

The necessary sample size for this study was calculated from an estimated percentage for ACADS genotypes homozygous or compound heterozygous for the ACADS variants and/or the c.1058C>T mutation of approximately 6% in the reference group, based on

<table>
<thead>
<tr>
<th>Table 1. ACADS genotypes in study populations</th>
<th>Current study</th>
<th>Nagan study</th>
<th>Previous study</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
</tr>
<tr>
<td>Total</td>
<td>909</td>
<td>100</td>
<td>694</td>
</tr>
<tr>
<td>511C/C</td>
<td>827</td>
<td>91.0</td>
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<tr>
<td>511T/C</td>
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<td>8.9</td>
<td>2</td>
</tr>
<tr>
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<td>534</td>
<td>59.7</td>
<td>221</td>
</tr>
<tr>
<td>625A/G</td>
<td>318</td>
<td>35</td>
<td>39</td>
</tr>
<tr>
<td>625A/A</td>
<td>48</td>
<td>5.3</td>
<td>39</td>
</tr>
<tr>
<td>1058C/C</td>
<td>903</td>
<td>99.3</td>
<td>2</td>
</tr>
<tr>
<td>1058T/C</td>
<td>6</td>
<td>0.7</td>
<td>2</td>
</tr>
<tr>
<td>Combinations</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1058T/C+625A/G</td>
<td>1</td>
<td>0.1</td>
<td>2</td>
</tr>
<tr>
<td>625A/G+511T/C</td>
<td>18</td>
<td>2</td>
<td>7</td>
</tr>
</tbody>
</table>
previous studies.\textsuperscript{1,5,6} A percentage 3 times as high, i.e. 18\%, in the patient group was supposed to indicate an association between these specific ACADS genotypes and epilepsy. Using a two-tailed $\alpha$ = 0.05 and power = 80\% we estimated that a sample size of at least 65 patients and 260 reference subjects were required.

To estimate the association between the ACADS genotypes and epilepsy, the odds ratio was calculated. As methods for obtaining plasma and the time period between blood puncture and C4-C analysis were different between the epilepsy and reference group, the C4-C results were not compared between these 2 groups. In 311 blood spots from the reference group acylcarnitine analyses were performed in the first year after they were obtained (>5 years after heel puncture) and in 598 blood spots in the fifth year after they were obtained (>9 years after heel puncture). To compare C4-C values between 5-year-old blood spots and 9-year-old blood spots within each of the 14 screening districts in the reference group, the Mann-Whitney test was used.

### Results

#### Incidence of c.511C>T and c.625G>A ACADS gene variants and the c.1058C>T mutation in the Dutch population reference group

The distribution of genotypes determined for the c.511C>T variant was clearly in favour of the wild type alleles. In 81 of the 909 individuals (8.9\%) one copy of the c.511C>T

<table>
<thead>
<tr>
<th>Observed frequency (%)</th>
<th>Expected frequency (%)</th>
</tr>
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<tbody>
<tr>
<td><strong>Total</strong></td>
<td>100</td>
</tr>
<tr>
<td>511C/C</td>
<td>91.0</td>
</tr>
<tr>
<td>511T/C</td>
<td>8.9</td>
</tr>
<tr>
<td>511T/T</td>
<td>0.1</td>
</tr>
<tr>
<td>625G/G</td>
<td>59.7</td>
</tr>
<tr>
<td>625A/G</td>
<td>35</td>
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<tr>
<td>625A/A</td>
<td>5.3</td>
</tr>
<tr>
<td>1058C/C</td>
<td>99.3</td>
</tr>
<tr>
<td>1058T/C</td>
<td>0.7</td>
</tr>
</tbody>
</table>

**Table 2. Expected genotype frequencies calculated from the observed allele frequencies assuming Hardy-Weinberg equilibrium**
variant was identified and 1 individual (0.1%) was homozygous for this variant (Table 1). Homozygosity and heterozygosity for the c.625G>A variant was far more prevalent, with percentages of 5.3 and 35% respectively. In 18 of the 909 individuals (2%) compound heterozygosity for the 2 different variants was established. In 6 of the 909 individuals (0.7%) 1 copy of the c.1058C>T mutation was identified. One of these 6 also carried 1 copy of the c.625G>A gene variant.

Overall, the c.511C>T and c.625G>A ACADS variants and the c.1058C>T ACADS mutation were detected in homozygous and compound heterozygous forms in 68/909 (7.5%, 95% CI 5.9-9.4%) subjects from the Dutch population.

Expected genotype frequencies in the Dutch population assuming Hardy-Weinberg equilibrium

Based on the observed allele frequencies of 4.6, 22.8, and 0.3% for the c.511C>T and c.625G>A ACADS variant and c.1058C>T mutation respectively, expected genotype frequencies were calculated assuming Hardy-Weinberg equilibrium (Table 2).

Demographic data of the epilepsy study group

Data of 131 epilepsy patients (53 girls and 78 boys) were studied. Age at study inclusion ranged between 0 and 16 years (median 9 years). Age of first epileptic insult ranged from 0 to 6 years (median 3 years). In all patients, the epilepsy was defined as idiopathic.

<table>
<thead>
<tr>
<th>Table 3. ACADS genotypes in the epilepsy and reference group</th>
</tr>
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<tbody>
<tr>
<td><strong>Epilepsy group</strong></td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>511C/C</td>
</tr>
<tr>
<td>511T/C</td>
</tr>
<tr>
<td>511T/T</td>
</tr>
<tr>
<td>625G/G</td>
</tr>
<tr>
<td>625A/G</td>
</tr>
<tr>
<td>625A/A</td>
</tr>
<tr>
<td>1058C/C</td>
</tr>
<tr>
<td>1058T/C</td>
</tr>
</tbody>
</table>

| Combinations | | |
| 1058T/C+625A/G | 0 | 0 | 1 | 0.1 |
| 625A/G+511T/C | 2 | 1.5 | 18 | 2 |
| 511T/T, 625A/A, 1058T/C+625A/G, 625A/G+511T/C | 12 | 9.2* | 68 | 7.5* |

* Not significantly different (OR 1.3, 95% CI 0.7-2.4)
Development was normal in 92/131 (70%) patients and 39/131 (30%) had delayed cognitive and/or motor function.

Incidence of c.511C>T and c.625G>A ACADS gene variants and the c.1058C>T mutation in the epilepsy study group and comparison with the reference group

In 12 of the 131 patients (9.8%) one copy of the c.511C>T variant was identified, but none of the epilepsy patients was homozygous for this variant (Table 3). The c.625G>A variant was identified in heterozygous form in 48 (36.1%) and in homozygous form in 10 (7.5%) epilepsy patients. One of the patients (0.8%) carried 1 copy of the c.1058C>T mutation. Overall, the c.511C>T and c.625G>A ACADS variants and the c.1058C>T ACADS mutation were detected in homozygous and compound heterozygous forms in 12/131 (9.2%, 95% CI 4.8-15.5%) epilepsy patients. This was comparable to the reference group (7.5%, Table 3). Within the epilepsy group, these combined ACADS genotypes were established in

Figure 3. C4-Carnitine in 311 5-year-old blood spots from reference subjects. The arrow points towards the result of the only reference subject with an ACADS genotype compound heterozygous for the c.625G>A variant and c.1058C>T mutation. Abbreviations: FL, Flevoland; RD, Rotterdam; NH, Noord-Holland; AD, Amsterdam; UT, Utrecht; GL, Gelderland; ZH, Zuid-Holland; NB, Noord-Brabant; OV, Overijssel; LB, Limburg; ZL, Zeeland; FR, Friesland; GN, Groningen; DR, Drenthe.
4/39 (10%, 95% CI 2.9-24.2%) patients with developmental delay and in 8/92 (8.7%, 95% CI 3.8-16.4%) patients without developmental delay.

C4 carnitine levels in the Dutch population and epilepsy study group
C4-C values in the 9-year-old blood spots were significantly (p<0.0001) lower in 12 out of 14 screening districts and significantly higher (p<0.0001) in 2 out of 14 screenings districts, compared to the values in 5-year-old blood spots from the same districts. Therefore, the results of the analyses of the 9-year-old blood spots were taken to be unreliable and are not included in this paper.

In the collection of 311 5-year-old blood spots, 11 were found to have C4-C values above the upper limit of 0.87 μmol/L (Figure 3). The highest C4-C value was established in the only reference subject with one 1 copy of the c.625G>A variant in combination with 1 copy of the c.1058C>T mutation. None of the other 10 subjects with increased C4-C values carried more than 1 copy of an ACAD5 variant or the c.1058C>T mutation.

None of the epilepsy patients was found to have a blood spot C4-C concentration above the upper limit of 0.87 μmol/L (Figure 4). Therefore an elevation in C4-C concentration in blood spots was not identified more often in the patient compared to the reference group.
Discussion

The current study is the first one investigating the prevalence of the c.511C>T ACADS variant and the c.1058C>T ACADS mutation in the Netherlands. In addition, it is the first study investigating a potential association between SCADD and one of its most frequently associated symptoms: epilepsy.

A number of 131 patients and 909 reference subjects were included, resulting in >98% power to detect a difference of 12% between the 2 groups.

Homozygosity and heterozygosity for the c.511C>T ACADS variant in the Dutch population (0.1 and 8.9% respectively) were found to be comparable to numbers in the US population (Table 1). The prevalence of the most common ACADS variant, the c.625G>A, in the Netherlands, has been investigated previously. The current study provided similar results (5.3 and 35% respectively, Table 1). As these results also are comparable to numbers in the US population, it appears that both ACADS variants, which are supposed to confer susceptibility for the development of SCADD, show a proportional representation throughout the Western world. This implies that every 1:14 Western newborns should be considered to be susceptible to the development of SCADD.

In the Netherlands with an annual birth rate of 180 000, this would correspond to approximately 12 500 newborns with supposed susceptibility for SCADD each year.

In addition we investigated the prevalence of the c.1058C>T mutation, which is supposed to represent a Dutch founder mutation. The results of the current study do provide evidence for a founder mutation as 0.7% percent of the reference group was identified with 1 c.1058C>T copy, all patients carrying the c.1058C>T mutation were of Dutch ancestry, and as this mutation has not been reported in patients without Dutch ancestors. Based on the established frequencies for this Dutch mutation and the ACADS variants, a birth-prevalence as high as 1:1000 for an ACADS genotype, which is compound heterozygous for 1 of the ACADS variants and the c.1058C>T mutation, can be calculated. This ACADS genotype has been associated with biochemical features (increased C4-C and/or EMA) of SCADD in all previously reported cases and was associated with the highest C4-C concentration in the current study. For the Netherlands this would imply that in addition to approximately 12 500 newborns with supposed susceptibility for SCADD, 180 newborns with SCADD would be born every year. The results of our study demonstrate that the combined prevalence of all SCADD related genotypes in the Netherlands amounts to at least 1:1000, which is significantly higher than our previous estimate of 1:3300. This is in strong contrast with the less than 40 patients with SCADD that have been diagnosed in the Netherlands within the last decades.

Failure to diagnose SCADD may be explained if SCADD often presents with clinical signs and symptoms generally not leading to metabolic studies in urine or plasma for increased EMA and/or C4-C concentrations. As epilepsy appears to be a common clinical symptom in SCADD, and as uncomplicated epilepsy in childhood is usually not followed by diagnostic tests for metabolic diseases, a high prevalence of SCADD in patients with
unexplained epilepsy might in part explain the high number of unrecognized SCADD patients.

We therefore investigated the presence of the most common \textit{ACADS} genotypes (representing 61\% percent of the \textit{ACADS} genotypes in the Dutch SCADD patient group) and the presence of an increased C4-C (as present in almost all Dutch SCADD patients with at least one \textit{ACADS} mutation) in pediatric patients with epilepsy of unknown origin and reference subjects. However, our study failed to demonstrate an association between homozygosity or compound heterozygosity for the c.511C>T and c.625G>A \textit{ACADS} gene variant and/or the Dutch c.1058C>T \textit{ACADS} mutation and/or increased C4-C in blood spots on the one hand, and epilepsy of unknown origin in childhood on the other hand.

A first limitation of our study, intrinsic to our study design, is that we compared \textit{ACADS} genotypes rather than SCADD defined by the presence of increased biochemical characteristics (increased C4-C and/or EMA) in combination with an \textit{ACADS} genotype. A second limitation is that we cannot rule out an association of epilepsy with the studied \textit{ACADS} genotypes with an OR smaller than 2.4. However, given the results of our study, the probability of an OR larger than 2.4 is 2.5\% only, and therefore not very likely.

In conclusion, the results of our study show that the prevalence of SCADD in the Netherlands is at least 1 in every 1000 newborns, but do not provide evidence for an association between SCADD and epilepsy. Therefore, SCADD does not seem to be an important risk factor for the development of epilepsy and metabolic investigations aimed to diagnose SCADD in epilepsy of unknown origin in childhood cannot be recommended.

\textbf{Acknowledgments}

This work was supported by Sigma-Tau B.V., “Metakids” (www.metakids.nl, the Dutch association for research on metabolic disorders), “Stichting Emma Kinderziekenhuis”, and “Stichting A.C. Thomsenfonds”. The authors thank the epilepsy patients and their parents for their participation in this study; the RIVM for providing the neonatal screening cards; dr. H.Geesink, Prof. dr. B.T. Poll-The and dr. W.C.G. Plandsoen for participating in this study; and Hetty Wijnia, Anette van Steenbergen and Mieke Stolwijk from the Stichting Epilepsie Instellingen Nederland and Thessa Westphal and Klaske Honig from the department of pediatrics/metabolic diseases of the AMC for their administrative support.
References


Chapter 9

General discussion and recommendations for clinical practice
General discussion and recommendations for clinical practice

In this final chapter, the results of the studies presented in the preceding chapters will be discussed. In addition, a diagnostic guideline on increased ethylmalonic acid (EMA) and C4- Carnitine (C4-C), the biochemical characteristics of short-chain acyl-CoA dehydrogenase (SCADD) will be presented.

The following aspects of SCADD will be discussed:
1. Clinical aspects
2. Biochemical, genetic and epidemiological aspects
3. Pathophysiological and therapeutic aspects
4. Clinical relevance
5. Guideline for the diagnostic work-up in the case of increased EMA and/or C4-C

1. Clinical aspects

A remarkable spectrum of clinical signs and symptoms has been attributed to SCADD since the very first SCADD patient was reported in 1987. Developmental delay is by far the most frequently reported symptom, followed by epilepsy, hypoglycemia, and hypotonia. In addition, behavioral disorders, dysmorphic features, myopathy, metabolic acidosis, acute fatty liver of pregnancy, failure to thrive, hypertonia, lethargy, fatigue, exercise intolerance, microcephaly, scoliosis, optic atrophy, feeding problems, and vomiting have all been reported in patients diagnosed with SCADD, albeit less frequently. Certain symptoms have been reported by specific centers only. Behavioral disorders were reported almost solely in the patients from one specific metabolic center participating in our study on Dutch SCADD patients, while multi-minicore myopathy was reported in association with SCADD only in a study of patients of Ashkenazi Jewish descent by Tein and colleagues. This may be caused by a correlation between specific symptoms and certain ACADS mutations that may be more prevalent in people with certain genetic backgrounds. Alternatively, the observed differences in the prevalence of rare symptoms may be caused by ascertainment bias, due to the fact that metabolic studies for SCADD are performed on different clinical grounds by different metabolic centers.

It is remarkable that the severity of the clinical spectrum of SCADD patients reported in literature has changed considerably since the description of the very first patients. As mentioned in the introduction, 2 out of the first 4 genetically identified SCADD patients were reported to have died at young age. However, one of the 2 patients that was reported to have died, and who is in fact the very first patient reported with SCADD,
was later reported in a conflicting publication to have shown a full clinical recovery, to study at a prestigious university, and to be a competitive equestrian at the age of 19. Of the >180 patients subsequently diagnosed and reported with SCADD, only one has been reported to have died, and many were reported to have recovered completely from the initial clinical symptoms. The 2 patients finally reported to have died both carried ACADS variants only and died from respiratory failure due to severe muscular hypotonia. Because it has been established that approximately 6% of the general population carries these variants on both ACADS alleles (chapters 2 and 8), the presumed association between SCADD and these clinical histories might be disputed. Furthermore, the clinical spectrum has been expanded by reports on asymptomatic family members with SCADD (chapter 3) and reports stating that almost all newborns diagnosed by screening have remained symptom free.

2. Biochemical, genetic, and epidemiological aspects

Data on EMA excretion, C4-C concentrations, and ACADS genotypes of all Dutch SCADD patients known at the time of study were reported in chapter 3. A clear association between both EMA excretion and C4-C concentrations and the ACADS genotype could be detected. The highest EMA and C4-C levels were observed in patients with mutations on both ACADS alleles (mut/mut group) ranging from 4-9 times the upper reference value for C4-C and 12-40 times the upper reference value for EMA. The lowest levels were observed in patients carrying ACADS variants (var/var group) on both alleles in the absence of a mutation. In this group, C4-C and/or EMA were only moderately elevated, ranging from normal values to 1.5 times the upper reference values for C4-C and 4 times the upper reference value for EMA. As a consequence, in patients with a var/var genotype the diagnosis of SCADD might easily be missed due to only minimal changes in the concentration of EMA and/or C4-C. Furthermore, because EMA increases during fasting (chapter 4) and C4-C increases during exercise (chapter 6) and FAD status appears to influence the concentration of EMA and C4-C (chapter 5), external factors may influence these diagnostic features. Therefore, we hypothesize that within the population homozygous for ACADS variants, i.e. 6% of the general population, the presence of mildly increased EMA or C4-C levels may depend on other factors including the period of fasting preceding the sampling of blood and collection of urine, the level of exercise preceding these studies, and the amount of riboflavin in the diet. In contrast to the detected association between genotype and biochemical phenotype, no correlation could be established between genotype and clinical phenotype. Moreover, most of the severely affected patients belonged to the var/var group, associated with only minimally increased EMA and/or C4-C (chapter 3). As stated previously, this was the case for the only 2 SCADD patients reported in literature to have died.
Table. Newly identified and previously reported mutations and variants in the ACADS gene

<table>
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<tr>
<th>Reference</th>
<th>DNA mutation*</th>
<th>Coding effect*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutations</td>
<td></td>
<td></td>
</tr>
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<td>Gregersen et al., 23 2008</td>
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**Variants**

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*Nomenclature according to the human genome variation society*
The number of reported ACADS mutations has expanded steadily in the last decade and 67 mutations have been reported so far. \textsuperscript{3,6,9,10,13,15,19,22-24} We report 12 new rare mutations (Table), resulting in a total number of 79, in addition to the 2 common ACADS variants. An interesting observation made by both Pedersen and Gregersen and colleagues, is that to date no SCADD patients with null mutations on both alleles have been reported.\textsuperscript{6,23} However, this may not be surprising considering that to date, 186 SCADD individuals worldwide have been reported and rare mutations on both ACADS alleles have been detected in only 30 (16\%) of them. Within this group of 30 persons, 19 different rare mutations were identified. These represent only 28\% of the total number of 67 rare mutations reported. Therefore, 48 (72\%) rare mutations, including the 7 null mutations, have not yet been established on both ACADS alleles of one individual.

SCADD was originally considered to be a rare disorder. In \textbf{chapters 2, 3, and 8}, the results of studies on the epidemiological aspects of SCADD were reported. First, we established the prevalence of the most common ACADS variant, c.625G>A. This variant was found to be present in homozygous and heterozygous form in as much as 5.6\% and 31.3\% of the Dutch population respectively (\textbf{chapter 2}). Secondly, based on the number of patients diagnosed in the Netherlands, combined with newborn studies in the U.S., we initially calculated a birth-prevalence of SCADD of approximately 1:3300 (\textbf{chapter 3}). In addition, we established the prevalence of the other known ACADS variant, c.511C>T, and found it to be present in homozygous and heterozygous form in 0.1\% and 8.9\% of the Dutch population, respectively. Furthermore, we investigated the prevalence of the most common Dutch ACADS mutation, c.1058C>T, which was present in a heterozygous form in 0.7\% of the Dutch population. Based on these numbers, we were able to calculate a birth-prevalence as high as 1:1000 for SCADD based exclusively on compound heterozygosity for one of the ACADS variants and the c.1058C>T mutation in the Netherlands. This implies that SCADD is indeed a very common inborn error of metabolism.

3. Pathophysiological and therapeutic aspects

\textbf{Chapters 4, 5, and 6} provide information on the pathophysiological as well as therapeutic aspects of SCADD. Ketogenesis in SCADD patients was found to be normal and fasting induced hypoglycemia only occurred in those SCADD patients who had a history of hypoglycemia, resembling idiopathic ketotic hypoglycemia in all (\textbf{chapter 4}). Stimulating mitochondrial β-oxidation by fasting, fat-loading or exercise affected the level of the 2 most important SCADD metabolites, with observed increases in EMA excretion during fasting and fat-loading and observed increases in plasma C4-C concentration during exercise. These latter observations suggest that there may well be alternative pathways for short-chain fatty acids in SCADD that differ between liver and muscle tissue, possibly based on higher intramitochondrial carnitine concentrations in muscle compared to liver.
As the C4-C or EMA increase was the only abnormality observed, the accumulation of toxic metabolites appears to be the most likely mechanism to be involved in the pathophysiology of SCADD. This has indeed been suggested by animal studies. Since the late eighties, 3 different kinds of studies in animal models have been reported. First, a spontaneous animal model for SCADD was discovered that represented SCADD with null mutations on both alleles in BALB/cByJ mice. These SCADD mice were found to develop glucose concentrations at 50% of controls as well as fatty livers upon fasting, to be cold-sensitive, and to have a marked slowing in theta frequency during paradoxical (REM) sleep. Apart from the risk of hypoglycemia during fasting, which could not be experimentally verified for human SCADD (Chapter 4), the phenomena observed in SCADD mice have not been investigated in human SCADD patients. However, because the BALB/cByJ mice represent a knock-out model, the comparison with SCADD patients, of which none have null mutations on both alleles, might not be appropriate. Furthermore, a different mouse model that appears to be more relevant to the human situation, was developed by Holm and colleagues. Expression of SCAD proteins, including human wild-type, the c.625G>A variant and the c.319C>T mutation, was established after transfer of human ACADS to SCADD mice, allowing functional testing of SCAD proteins in vivo. A subsequent study in offspring of these transgenic mice and the original SCADD knock-out mice showed expression of human wild-type SCAD only, suggesting that the other 2 human folding variants were rapidly eliminated. However, none of the transgenic lines showed any disease phenotype or change in mortality when compared to control mice. Finally, several animal studies focusing on the toxicity of EMA have been performed. The latest study on this subject showed that high sustained brain EMA during early development could compromise biochemical pathways involved in learning/memory processes. In this regard, it may be important to mention that it has been established recently that in ethylmalonic encephalopathy (EE), a devastating disorder with very high EMA levels, sulfide toxicity actually seems to be responsible for several clinical features and inhibition of SCAD as well as COX activity. In addition, our observation that SCADD patients with the highest levels of EMA and C4-C did not show a more severe phenotype than the patients with lower levels (chapter 3) argues against a pathological role of these metabolites, at least under physiological conditions in humans.

Another factor that might be involved in the pathophysiological mechanisms of SCADD is the FAD status. Our results suggest that a relatively low FAD concentration may be involved in the development of biochemical features of SCADD (chapter 5). This resembles the results of studies in the SCADD mouse, which showed an increase of certain relevant metabolites upon a riboflavin-deficient diet. However, even though we demonstrated that high-dose riboflavin treatment leads to biochemical improvement in a subgroup of SCADD patients, our studies did not provide evidence for any improvement in clinical parameters.

The therapeutic consequences that emerged in our studies can be summarized as follows: unless the clinical history suggests hypoglycemia, the diagnosis of SCADD should
not routinely be followed by a fasting test or late evening meal (chapter 4) and general treatment with riboflavin cannot be recommended (chapter 5).

4. Clinical relevance

The results of our studies described in chapters 3, 4, 7, and 8 provide extensive evidence for a lack of clinical relevance of SCADD. Based on these results, SCADD can be classified as a very common inborn error of metabolism with remarkably few patients diagnosed compared to its calculated prevalence in the community (chapter 8). Because this might be due not only to a lack of clinical relevance, but also to the omission of metabolic investigations in patients with SCADD-associated symptoms, the presence of the most common ACADS genotypes was studied in patients with epilepsy of unknown origin and reference subjects. However, this study failed to reveal evidence for a correlation between SCADD and epilepsy (chapter 8). In addition, symptoms in SCADD patients often ameliorated and even disappeared spontaneously during follow-up, and in several cases, symptoms initially attributed to SCADD could later be explained by other causes (chapter 3). Furthermore, SCADD relatives as well as almost all SCADD individuals diagnosed by newborn screening remained asymptomatic during follow-up (chapter 3). Finally, although we cannot exclude that SCADD might become clinically relevant under extreme circumstances like starvation and/or cold, the results of fasting and fat-loading studies did not suggest any pathophysiological significance of SCADD (chapter 4).

All of this information implies a lack of clinical significance of this disorder of short-chain fatty acid oxidation (FAO), which may therefore be even more appropriately referred to as a metabolic variant. It raises the question whether humans would actually be able to do without the metabolic efforts of the SCAD enzyme. Gregersen and Pedersen and colleagues speculated that null mutations on both ACADS alleles would be lethal. If this were true, there would be a huge clinical difference between individuals with ACADS mutations (including at least one missense type) on both alleles, which is associated with very high EMA and C4-C levels but frequently without clinical symptoms, and any currently not recognized individuals with null mutations on both alleles. This would imply that even the slightest amount of SCAD activity might be associated with a normal clinical phenotype but that no SCAD activity would be disastrous. Even if this were the case, it would not change the observations that SCADD is clinically irrelevant in the vast majority of known SCADD individuals, if not in all.

In chapter 3, we raised the issue that SCADD does not meet the current criteria for inclusion in newborn screening programs. All of our subsequent studies have further substantiated this notion. Partly based on the results described in chapter 3, SCADD has been excluded from newborn screening programs in Australia. However, SCADD is still screened for in the majority of U.S. states. Because false positive screening results...
may generate substantial anxiety in the parents and lead to more hospitalizations of the child,\textsuperscript{36} we are concerned that screening for SCADD might have unwanted effects. Results of long-term follow-up of SCADD newborns will certainly further expand the information on the clinical relevance of SCADD. However, due to the high cut-off levels for C4-C in newborn screening, only a selected group of SCADD individuals, i.e. predominantly those with \textit{ACADS} mutations on at least one allele, will be identified. As patients with this genotype were by far not the most severely clinically affected among the SCADD individuals (chapter 3), even follow-up of a cohort of current SCADD newborns will not provide the ultimate evidence on the relevance of this disorder. It therefore appears highly disputable to proceed with SCADD newborn screening based on this argument.

Finally, it will never be possible to completely rule out any contribution of SCADD to the development of clinical disease. However, it seems realistic to assume that it is highly likely that SCADD is clinically irrelevant. Therefore, SCADD may best be considered as a metabolic variant.

5. \textbf{Guideline for the diagnostic work-up in the case of increased EMA and/or C4-C}

In view of the lack of clinical relevance of SCADD, it may be questioned if any additional investigations should be performed to achieve a definitive diagnosis of SCADD once SCADD is suspected on the basis of increased EMA and/or C4-C. In this regard it is important to realize that the differential diagnosis of increased EMA and/or C4-C includes several other metabolic disorders, including several potentially severe inborn errors of metabolism, such as disorders of oxidative phosphorylation (OXPHOS) diseases, multiple acyl-CoA dehydrogenase deficiency (MADD) and EE. In addition, a disorder of less clear clinical significance, isobutyryl-CoA dehydrogenase deficiency (IBD), may biochemically mimic SCADD. For this reason, additional metabolic investigations should be performed if SCADD is suspected on the basis of increased EMA and/or C4-C in the presence of clinical signs and symptoms. Although OXPHOS disorders and MADD may generally be ruled out rather easy, more diagnostic efforts might be necessary to rule out EE and IBD.

A proposal for a practical guideline is presented in the figure. Once elevated EMA and/or C4-C are discovered, OXPHOS disorders and MADD should be considered when organic acid and acylcarnitine analyses reveal multiple other abnormalities.\textsuperscript{37,38} MADD is characterized by a permanently increased urine excretion of D-2-hydroxyglutaric acid as well as variable amounts of urine glutaric acid and even-chain dicarboxylic acids. Plasma acylcarnitines will reveal elevated straight-chain acylcarnitines with chain lengths C16-18. OXPHOS defects may display abnormal amounts of lactate and citric acid cycle intermediates in the urine and some patients have a generalized aminoaciduria. If C5-Carnitine (C5-C) is increased in addition to an increased EMA and/or C4-C, \textit{ETHE 1} sequencing should be performed.
to confirm the diagnosis of EE\textsuperscript{39}. If only EMA and/or C4-C are increased, repeating the measurements under non-stressed conditions is advised. Moderately increased EMA and/or C4-C are almost always associated with homozygosity for the \textit{ACADS} variants, and in these cases, EMA and/or C4-C may be inconsistently increased, especially when avoiding “metabolic stressors” such as prolonged fasting and exercise. If EMA and/or C4-C are found to be persistently elevated, further studies are advised. These additional studies are based on the assumptions that, if available, separation of C4-C from iso-C4-C differentiates between SCADD and IBD, EMA is normal in IBD\textsuperscript{40}, and that SCADD is the far most prevalent disorder involved. Finally, after the diagnosis of SCADD, patients and/or parents should be informed that SCADD is a metabolic variant, and further diagnostic studies for potential causes for the clinical signs and symptoms should be performed.

**Conclusion**

SCADD should be considered as a common metabolic variant. The \textit{ACADS} genotype is correlated with the biochemical phenotype, i.e. the degree of EMA and C4-C increase, and the latter can be influenced by the FAD status and external circumstances that stimulate FAO, particularly fasting. Considering SCADD as a metabolic variant implies that SCADD should not be included in newborn screening programs. In clinically identified patients with increased EMA and/or C4-C, clinically relevant disorders that are associated with these biochemical characteristics should be excluded, which may result in the diagnosis of SCADD as a consequence. The diagnosis of this metabolic variant should lead to appropriate counseling of the patient, parents, and family and further diagnostic studies to identify other potential causes of the clinical signs and symptoms.

**Figure.** Proposed guideline in the case of clinical signs and symptoms and increased ethylmalonic acid and/or C4-Carnitine. Abbreviations: EMA, ethylmalonic acid; C4-C, C4-carnitine; OA, organic acids; AC, acylcarnitines; C5-C, C5-carnitine; TCA, tricarboxylic acid cycle; \textit{ETHE}, gene coding for sulfide dioxygenase, responsible for ethylmalonic encephalopathy; OXPHOS, oxidative phosphorylation; DH, dehydrogenase; MADD, multiple acyl-CoA dehydrogenase deficiency; EE, ethylmalonic encephalopathy; \textit{ACADS}, acyl-CoA dehydrogenase short; \textit{ACAD8}, acyl-CoA dehydrogenase 8; IBD, isobutyryl-CoA dehydrogenase deficiency; SCADD, short-chain acyl-CoA dehydrogenase deficiency.
Conclusion

SCADD should be considered as a common metabolic variant. The ACADS genotype is correlated with the biochemical phenotype, i.e. the degree of EMA and C4-C increase, and the latter can be influenced by the FAD status and external circumstances that stimulate FAO, particularly fasting. Considering SCADD as a metabolic variant implies that SCADD should not be included in newborn screening programs. In clinically identified patients with increased EMA and/or C4-C, clinically relevant disorders that are associated with these biochemical characteristics should be excluded, which may result in the diagnosis of SCADD as a consequence. The diagnosis of this metabolic variant should lead to appropriate counseling of the patient, parents, and family and further diagnostic studies to identify other potential causes of the clinical signs and symptoms.
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Summary
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Short-chain acyl-CoA dehydrogenase deficiency (SCADD) is a relatively newly recognized autosomal recessive inborn error of metabolism, for which many issues still need to be elucidated. The SCAD enzyme participates in the final β-oxidation cycle of mitochondrial fatty acid oxidation (FAO), a pathway central to the provision of energy for the mammalian organism. SCADD is most frequently reported to be associated with neurological symptoms and/or hypoglycemia, although the spectrum of clinical symptoms is remarkably broad. Most SCADD patients carry common SCAD-encoding gene (ACADS) variants or ACADS variants in combination with an ACADS mutation. The ACADS variants are considered to confer susceptibility to development of clinical disease and their high prevalence suggests that SCADD might be far more prevalent than initially assumed. SCADD is biochemically characterized by increased levels of C4-carnitine (C4-C) in plasma and ethylmalonic acid (EMA) in urine. The pathophysiology of SCADD is still speculative and the efficacy of a potential treatment has never been systematically studied. Nevertheless screening for SCADD had been included in newborn screening programs in most United States and Australian states.

This thesis focuses on the clinical, biochemical, genetic, epidemiological, pathophysiological, and therapeutic aspects of SCADD in order to increase knowledge of this intriguing disorder and to provide better care for the patients.

In Chapter 2, the results of a study on the prevalence of the c.625G>A ACADS variant in the Netherlands as well as the correlation of C4-C levels with the c.625G>A variant are reported. The screening cards of 1036 newborns were analyzed, and homozygosity and heterozygosity for the c.625G>A variant was established for 5.5% and 31.3% of these individuals, respectively. No significant differences were found for C4-C concentrations or for ratios of C4-C to free carnitine in blood spots among newborns who were homozygous, heterozygous, or non-carriers for this ACADS variant. Therefore, a high frequency of the c.625G>A ACADS variant in the Dutch population, but no correlation with significantly increased C4-C levels in blood spots taken between the 5th and 8th days of life was demonstrated. This latter observation might be the result of the relatively late timing of newborn screening in the Netherlands, which implies that some FAO disorders may be missed at that stage. If the c.625G>A variant is associated with clinical SCADD, the high frequency of the variant suggests a possible involvement of SCADD in the pathogenesis of common disorders, probably in relation to other genetic and/or environmental factors. However, homozygosity for the c.625G>A ACADS variant might be only a biochemical phenomenon, representing a non-disease.

Chapter 3 provides extensive data on clinical, biochemical, and genetic features of SCADD based on a group of 31 Dutch SCADD patients and their 8 SCADD relatives. In addition, these data are used to discuss newborn screening for SCADD.
A birth-prevalence of at least 1:50,000 was calculated, which suggested that SCADD is more common than previously assumed. Most patients presented before the age of 3 years, with non-specific, generally uncomplicated and often transient symptoms. Developmental delay, epilepsy, behavioral disorders, and hypoglycaemia were the most frequently reported. The ACADS genotype, subdivided into mutation/mutation (mut/mut), mutation/variant (mut/var) and variant/variant (var/var) genotype groups showed a statistically significant association with EMA and C4-C levels but not with clinical characteristics. Seven out of 8 SCADD relatives were free of symptoms.

These data and data of newborn screening studies show that SCADD lacks clinical significance in many patients as well as individuals diagnosed by newborn screening and show that it is not possible to differentiate between diseased and non-diseased individuals. SCADD therefore does not meet major newborn screening criteria and is not suited for inclusion in newborn screening programs at the present time.

Chapter 4 provides insight into the pathophysiological consequences of SCADD through a retrospective study of 15 fasting and 6 fat-loading tests in 15 SCADD patients. Three patients developed hypoglycemia during fasting and all of these had originally presented with hypoglycemia. The EMA excretion increased in response to fasting and fat-loading, and plasma C4-C remained stable. Concentrations of all other studied metabolites in plasma and urine, including those related to ketogenesis, and all relevant hormones remained normal during all tests. Test results did not differ between the patients with a mut/mut, mut/var, and var/var genotype.

The metabolic profiles of the 3 patients who developed hypoglycemia resemble those of patients with idiopathic ketotic hypoglycemia. Because an unexplained episode of hypoglycemia will generally be followed by a metabolic work-up and because SCADD is relatively prevalent, SCADD may very well be diagnosed coincidently and thus be causally unrelated to the hypoglycemia. If SCADD has other pathological consequences, the accumulation of potentially toxic metabolites such as EMA is most likely involved. However, the results indicate that there is no clear pathophysiological significance, irrespective of genotype. This further supports the claim that SCADD is not suited for inclusion in newborn screening programs.

Chapter 5 presents results of a study on flavin adenine dinucleotide (FAD) status and the effects of riboflavin supplementation in a prospective open-label cohort study involving 16 SCADD patients, subdivided into mut/mut, mut/var, and 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 as compared with the mut/mut group. Riboflavin treatment caused a decrease in EMA excretion in the mut/var group and a subjective clinical improvement in 4 patients from this group. However, this improvement persisted after treatment was stopped. 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. A clinically relevant effect of riboflavin could not be demonstrated and therefore general use of riboflavin cannot be recommended.

The biochemical response to exercise and the effects of high-dose riboflavin therapy on the observed response in 3 SCADD patients, all with mut/var genotypes and symptoms of exercise intolerance or fatigue, are presented in Chapter 6. C4-C concentrations in plasma increased in response to exercise, while EMA excretion remained stable. One patient, who responded clinically to riboflavin therapy, was retested during riboflavin therapy. During this test, the increases in plasma C4-C in response to exercise were not observed anymore. However, 2 years after stopping riboflavin therapy, C4-C was again found to increase in response to exercise in this patient, even though she was still without clinical symptoms. As C4-C increased in SCADD patients during exercise and EMA increased during fasting, preferred tissue-specific pathways might exist. In addition, high-dose riboflavin therapy may prevent C4-C increase during exercise but this does not appear to be related to any clinical effect in this particular patient.

Chapter 7 presents a review of the clinical aspects of SCADD. SCADD generally presents early in life and is most frequently associated with developmental delay, hypotonia, epilepsy, behavioral disorders, and hypoglycemia. However, these symptoms often ameliorate, they can disappear spontaneously during follow-up, and they were found to be unrelated to the ACADS genotype. In addition, in some cases symptoms initially attributed to SCADD could later be explained by other causes. Finally, SCADD relatives of SCADD patients as well as almost all SCADD individuals diagnosed by neonatal screening, remained asymptomatic during follow-up.

Based on these observations, one may question the clinical relevance of SCADD. We therefore searched the database of the laboratory for metabolic diseases in our centre for the most frequently reported symptoms of patients for whom metabolic studies were requested. These symptoms were comparable to those in the SCADD patients group, suggesting that the association might indeed be coincidental.

The potential lack of clinical consequences implies that the diagnosis SCADD should never preclude extension of the diagnostic workup for other potential causes of the observed symptoms and patients and parents should be clearly informed about the potential irrelevance of the disorder. Furthermore, to date, SCADD does not qualify for inclusion in newborn screening programs. More studies are needed to establish the relevance of SCADD and to solve the question of whether SCADD is involved in a multifactorial disease or if it represents a non-disease.

In view of the debate on the clinical significance of SCADD and to further estimate the extent of the Dutch SCADD population, a case-referent study among 131 pediatric patients with epilepsy and 909 anonymous newborns was performed (Chapter 8). The 2 ACADS variants and the most common Dutch ACADS mutation, c.1058C>T, were
detected in either homozygous or compound heterozygous forms in 9.2% of the epilepsy group and in 7.5% of the reference group. In none of the epilepsy patients C4-C was increased. These data imply that the birth-prevalence of SCADD with a mut/var genotype in the Netherlands is even higher than 1:1000 but provide no evidence for an association between SCADD and epilepsy. Therefore SCADD studies in epilepsy of unknown origin in childhood cannot be recommended.

Finally in chapter 9, it is concluded that SCADD should be considered a very common metabolic variant. The ACADS genotype correlates with the biochemical phenotype, i.e. the degree of EMA and C4-C increase, and the latter can be influenced by the FAD status and by external circumstances that stimulate FAO, particularly fasting. Considering SCADD as a metabolic variant implies that SCADD should not be included in newborn screening programs. A practical guideline in the case of elevated EMA and/or C4-C is presented. This guideline illustrates that for clinically identified patients with increased EMA and/or C4-C, clinically relevant disorders that are associated with these biochemical characteristics should be excluded. This may, as a consequence, result in the diagnosis of SCADD, after which patients and/or parents should be informed that SCADD is a metabolic variant and additional diagnostic studies for potential causes of the clinical signs and symptoms should be performed.
Samenvatting
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Short-chain acyl-CoA dehydrogenase deficiëntie (SCADD) is een relatief recent ontdekte autosomaal recessieve (wat inhoudt dat door beide ouders, die drager zijn van de erfelijke aanleg en daar niet ziek van zijn, de erfelijke aanleg moet worden doorgegeven om de stoornis te krijgen) aangeboren stoornis in de stofwisseling, waarvan nog veel aspecten opgehelderd moeten worden. Het SCAD enzym (lichaamseigen eiwit dat zorgt voor het omzetten van een bepaalde stof) neemt deel aan de laatste cyclus van de mitochondriale vetzuuroxidatie. Dit is het proces in de stofwisseling dat een grote rol speelt bij de productie van energie voor het lichaam en het op peil houden van de bloedsuiker spiegel in het geval van langdurig vasten. Zoals de naam aangeeft, vindt dit proces plaats in mitochondriën, onderdelen van de cel die beschouwd kunnen worden als de energiecentrales van de cellen, met als “grondstoffen” de vetzuren afkomstig uit vetweefsel.

SCADD wordt in de wetenschappelijke literatuur voornamelijk geassocieerd met neurologische symptomen en/of hypoglycemie (lage bloedsuiker), maar er worden ook vele andere symptomen bij beschreven. De meeste SCADD patiënten hebben of wel 2 zogenaamde varianten van het SCAD gen (ACADS; gen dat de erfelijke informatie voor het SCAD enzym bevat) ofwel een ACADS variant en een ACADS mutatie (een echte “fout” in het DNA). De 2 varianten komen vaak voor en hiervan wordt verondersteld dat ze minder ernstig zijn dan een mutatie en dat ze gepaard gaan met een verhoogde gevoeligheid voor het ontwikkelen van ziekteverschijnselen door SCADD. Het veelvuldig vóórkomen (prevalentie) van deze ACADS varianten suggereert dat SCADD misschien wel veel vaker voorkomt dan aanvankelijk werd verondersteld.

SCADD kan worden opgespoord door het vinden van verhoogde waarden van C4-carnitine (C4-C) in bloed en ethylmalonzuur (EMA) in urine, de 2 biochemische kenmerken van SCADD. Ten aanzien van de pathofysiologie (het ontstaan van ziekteverschijnselen) van SCADD wordt slechts gespeculeerd en potentiële behandelingen zijn nog nooit systematisch onderzocht. Toch worden pasgeboren in het grootste deel van de Verenigde Staten en Australië met behulp van de hielprik op SCADD gescreend.

Dit proefschrift richt zich op de klinische, biochemische, genetische, epidemiologische, pathofysiologische en therapeutische aspecten van SCADD, met als doel de kennis over deze intrigerende stoornis te vergroten en zodoende de zorg voor patiënten te verbeteren.

In Hoofdstuk 2 wordt de prevalentie van de meest voorkomende ACADS variant, de c.625G>A, in Nederland, samen met het verband tussen de aanwezigheid van deze variant en de hoogte van het C4-C beschreven. De hielprikkaarten van 1036 pasgeboren werden geanalyseerd. Hierbij werd de c.625G>A variant bij 5,5% homozygoot (2 keer het “foutje”, dus door beide ouders doorgegeven) en bij 31,3% heterozygoot (1 x het foutje, dus door 1 ouder doorgeven) vastgesteld. Er werd geen verschil gevonden tussen
de C4-C waarden bij pasgeborenen die homozygoet, heterozygoet of niet-dragers waren voor deze ACADS variant. De c.625G>A ACADS variant komt dus heel veel voor onder de Nederlandse bevolking, maar er kon geen verband worden aangetoond met verhoogde C4-C waarden in het bloed dat tussen de vijfde en achtste levensdag met de hielprik werd afgenomen. Deze laatste observatie zou het gevolg kunnen zijn van het relatieve late tijdstip waarop het hielprkonderzoek in Nederland plaats vindt (baby’s krijgen dan meestal alweer voldoende voeding binnen, waardoor er geen vetzuuroxidatie hoeft plaats te vinden om energie te leveren). Dit suggereert dat sommige vetzuuroxidatie stoornissen gemist zouden kunnen worden op dit tijdstip. Als de c.625G>A variant in verband zou staan met klinische verschijnselen van SCADD, dan suggereert de hoge prevalentie van deze ACADS variant dat SCADD misschien wel een rol speelt bij veel voorkomende klinische verschijnselen. Waarschijnlijk spelen daarbij dan ook andere genetische en/of omgevingsfactoren een rol. Echter, het homozygoet aanwezig zijn van de c.625G>A ACADS variant zou ook uitsluitend een biochemisch fenomeen kunnen betreffen, overeenkomend met een zogenaamde “non-disease”.

**Hoofdstuk 3** biedt uitgebreide informatie over klinische, biochemische en genetische kenmerken van SCADD gebaseerd op een groep van 31 Nederlandse SCADD patiënten en hun 8 SCADD familieleden. Daarnaast wordt deze informatie gebruikt voor het bediscussiëren van de hielprk screening op SCADD.

Een geboorteprevalentie van minstens 1:50.000 werd berekend, wat suggereert dat SCADD vaker voorkomt dan aanvankelijk werd verondersteld. De meeste patiënten presenteerden zich voor de leeftijd van 3 jaar, met aspecifieke, over het algemeen ongecompliceerde en vaak voorbijgaande symptomen. Ontwikkelingsachterstand, epilepsie, gedragstoornissen en hypoglycemie werden het meest frequent gerapporteerd. Het ACADS genotype, onderverdeeld in mutatie/mutatie (mut/mut), mutatie/variant (mut/var) en variant/variant (var/var) genotype groepen, was statistisch significant geassocieerd met de hoogte van het EMA en C4-C, maar niet met klinische kenmerken. Zeven van de 8 SCADD familieleden hadden geen symptomen.

Deze gegevens en gegevens van hielprk screening studies laten zien dat SCADD bij vele patiënten en individuen die werden gediagnosticeerd door middel van de hielprk screening geen klinische betekenis heeft. Daarnaast maakt dit duidelijk dat het niet mogelijk is om op basis van de hielprkuitslag onderscheid te maken tussen individuen die symptomen zullen ontwikkelen en individuen die symptoomvrij zullen blijven. SCADD voldoet daarom niet aan de belangrijkste criteria voor de hielprk screening en is zodoende op dit moment niet geschikt voor opname in hielprk screening programma’s.

**Hoofdstuk 4** biedt inzicht in de pathofysiologische consequenties van SCADD. Hiervoor werden 15 vastentesten (waarbij afhankelijk van de leeftijd gedurende 22 of 46 uur niet werd gegeten en alleen water werd gedronken) en 6 vetbelastingstesten (waarbij ‘s ochtends in nuchtere toestand zonnebloemolie werd ingenomen) bij 15 SCADD
patiënten geanalyseerd. Drie patiënten, die zich allen hadden gepresenteerd met een hypoglycemie, ontwikkelden een hypoglycemie tijdens de vastentest. De EMA uitscheiding nam tijdens de test toe en het C4-C bleef stabiel. De overige uitslagen van relevant stofwisselingsonderzoek, waaronder de productie van ketonlichamen (energie die bij de vetzuuroxidatie in de lever wordt geproduceerd en door met name de hersenen gebruikt wordt als brandstof) en hormonaal onderzoek waren gedurende alle vastentesten en vetbelastingstesten niet afwijkend. De testresultaten verschillen niet tussen de patiënten met een mut/mut, mut/var en var/var genotype.

De stofwisselingsprofielen tijdens de vastentesten van de 3 patiënten met een hypoglycemie komen overeen met die van patiënten met een zogenaamde idiopathische ketotische hypoglycemie (hypoglycemie die vooral bij peuters/kleuters optreedt en waarvoor geen oorzaak kan worden aangetoond). Aangezien bij een hypoglycemie zonder bekende oorzaak meestal stofwisselingsonderzoek wordt uitgevoerd en omdat SCADD relatief vaak voorkomt, zou SCADD net zo goed per toeval kunnen worden vastgesteld bij deze patiënten met hypoglycemie en zodoende oorzakelijk niet gerelateerd hoeven te zijn aan de hypoglycemie. Als SCADD andere pathologische consequenties zou hebben, dan zou de ophoping van potentieel schadelijke stofwisselingsproducten zoals EMA het meest voor de hand liggen. Echter, het verder normale stofwisselingsprofiel maakt duidelijke pathofysiologische relevantie niet waarschijnlijk. Dit onderschrijft wederom de bewering dat SCADD niet geschikt is voor opname in hielprikscreening programma’s.

**Hoofdstuk 5** geeft de resultaten weer van een studie naar de flavine adenine dinucleotide (FAD) status en de effecten van hooggedoseerde riboflavine suppletie bij 16 SCADD patiënten, onderverdeeld in mut/mut, mut/var en var/var genotype groepen. Riboflavine (vitamine B2), wordt na opname in het lichaam omgezet in FAD. FAD is de co-factor (nodig voor de werking) van het SCAD enzym. Bloed FAD waarden waren normaal bij alle patiënten voor start van de suppletie, maar duidelijk lager bij de mut/var en var/var groepen in vergelijking met de mut/mut groep. Riboflavine therapie resulteerde in een afname van de EMA excretie in de mut/var groep en een subjectieve klinische verbetering bij 4 patiënten uit deze groep. Echter, deze verbetering bleef bestaan nadat de behandeling was gestopt. Deze resultaten laten zien dat hooggedoseerde riboflavine behandeling de biochemische kenmerken van SCADD kan verbeteren, ten minste bij patiënten met een mut/var genotype en lage FAD waarden. Een klinisch relevant effect van riboflavine kon niet worden aangetoond en daarom kan gegeneraliseerde riboflavine therapie bij SCADD niet worden aanbevolen.

De biochemische respons op inspanning (getest tijdens 60 minuten fietsen) en de effecten van hooggedoseerde riboflavine therapie op deze respons bij 3 SCADD patiënten, allen met een mut/var genotype en symptomen van inspanningsintolerantie of moeheid worden gepresenteerd in **Hoofdstuk 6**. C4-C waarden namen toe in respons op inspanning, terwijl de EMA excretie stabiel bleef. Eén patiënt, met subjectieve klinische
verbetering op riboflavine, werd opnieuw getest gedurende riboflavine therapie, waarbij inspanningsgeïnduceerde toename van C4-C niet meer werd waargenomen. Echter, 2 jaar na het staken van de riboflavine behandeling werd wederom een toename van C4-C vastgesteld in respons op inspanning bij deze patiënt, terwijl de symptomen niet waren teruggekeerd. Aangezien het C4-C toenam bij SCADD patiënten gedurende inspanning (waarbij vooral de stofwisseling in de spier is geactiveerd) en het EMA toenam gedurende vasten (waarbij vooral de stofwisseling in de lever is geactiveerd), lijken er weefselspecifieke voorkeursroutes te bestaan. Daarnaast lijkt hooggedoseerde riboflavine therapie te kunnen voorkomen dat C4-C toeneemt gedurende inspanning, maar dit lijkt niet gerelateerd te zijn aan een klinisch effect in deze specifieke patiënt.

Hoofdstuk 7 bevat een overzichtsartikel waarin de klinische aspecten van SCADD op een rijtje worden gezet. SCADD presenteert zich hoofdzakelijk in de eerste levensjaren en vooral ontwikkelingsachterstand, verlaagde spierspanning, epilepsie, gedragsstoornissen en hypoglycemie worden als klinische verschijnselen beschreven. Bij follow-up neemt de ernst van deze symptomen echter meestal af en soms verdwijnen de symptomen spontaan. Daarnaast blijken ze niet gerelateerd te zijn aan het ACADS genotype (onderverdeeld in mut/mut, mut/var en var/var groepen). Verder waren er in een aantal gevallen andere oorzaken die de symptomen, waarvan aanvankelijk gedacht werd dat ze werden veroorzaakt door SCADD, konden verklaren. Tenslotte bleven vrijwel alle SCADD familieleden van SCADD patiënten en SCADD individuen die gediagnosticeerd werden bij de hielprikscreening asymptomatisch gedurende follow-up.

Op basis van deze observaties kan men de klinische relevantie van SCADD in twijfel trekken. Wij raadpleegden daarom de database van het laboratorium voor stofwisselingsziekten in ons centrum. Daarbij kwam naar voren dat de meest frequent gerapporteerde symptomen bij patiënten waarbij stofwisselingsonderzoek werd aangevraagd overeenkomen met de symptomen van de SCADD patiënten, wat suggereert dat het verband inderdaad op toeval berust.

Het potentiële gebrek aan klinische consequenties van SCADD impliceert dat de diagnose SCADD nooit een uitbreiding van het diagnostisch onderzoek naar andere mogelijke oorzaken van de geobserveerde symptomen in de weg zou mogen staan. Daarnaast dienen patiënten en ouders duidelijk geïnformeerd te worden over de mogelijke klinische irrelevante van deze stoornis. Bovendien maakt dit nog eens duidelijk dat SCADD niet geschikt is voor opname in hielprikscreening programma’s. Aanvullende studies zijn nodig om de relevantie van SCADD verder te verduidelijken en de vraag op te lossen of SCADD is betrokken bij een multifactorië ziekte of dat het een “non-disease” betreft.

Met het oog op de discussie over de klinische relevantie van SCADD en om een beter beeld te verkrijgen van de omvang van de Nederlandse SCADD populatie, werd een studie uitgevoerd waarbij 131 kinderen met epilepsie werden vergeleken met 909 anonieme pasgeboren (referentie groep) (Hoofdstuk 8). Hierbij werden de 2 ACADS varianten
en de meest voorkomende Nederlandse ACADS mutatie, c.1058C>T, homozygooot of samengesteld heterozygooot (2 verschillende varianten of een variant en de mutatie) vastgesteld bij 9,2% van de epilepsie groep en 7,5% van de referentie groep. Bij geen van de epilepsie patiënten was het C4-C verhoogd. Op basis van deze resultaten kan gesteld worden dat de geboorteprevalentie van SCADD met een mut/var genotype in Nederland nog hoger is dan 1:1000 maar dat geen verband kan worden aangetoond tussen SCADD en epilepsie. Er is daarom geen reden voor diagnostiek naar SCADD bij kinderen met epilepsie met onbekende oorzaak.

Tenslotte wordt in hoofdstuk 9 geconcludeerd dat SCADD niet beschouwd zou moeten worden als een stofwisselingsziekte, maar als een veel voorkomende stofwisselingsvariant. Het ACADS genotype correleert niet met klinische verschijnselen, maar wel met de hoogte van EMA and C4-C, en dit kan beïnvloed worden door de FAD status en door externe omstandigheden die de vetzuuroxidatie stimuleren, in het bijzonder vasten. Het beschouwen van SCADD als een stofwisselingsvariant impliceert dat SCADD niet in hielprikscreening programma’s zou moeten worden opgenomen. In dit hoofdstuk wordt een praktische richtlijn voor het geval van een verhoogd EMA en/of C4-C gepresenteerd. Deze richtlijn illustreert dat bij klinisch geïdentificeerde patiënten met een verhoogd EMA en/of C4-C, klinisch relevante stoornissen, die eveneens gepaard gaan met deze biochemische karakteristieken, uitgesloten zouden moeten worden. Dit kan resulteren in de diagnose SCADD, waarna aan patiënten en/of ouders zal moeten worden uitgelegd dat SCADD beschouwd dient te worden als een stofwisselingsvariant en dat verder aanvullend onderzoek naar mogelijke oorzaken van de symptomen zal moeten plaats vinden.
Korte samenvatting

De resultaten van de in dit proefschrift beschreven studies naar een aangeboren defect in de afbraak van kort- keten vetzuren (Short-chain acyl-CoA dehydrogenase deficiëntie (SCADD)) laten zien dat deze stofwisselingsstoornis bijzonder vaak voorkomt onder de Nederlandse bevolking. Berekent wordt dat in Nederland minimaal 180 kinderen per jaar met deze stoornis geboren worden. Opvallend is dat echter slechts bij een heel klein deel van deze kinderen de diagnose SCADD gesteld wordt, meestal op basis van symptomen die leiden tot stofwisselingsonderzoek. Daarbij gaat het vooral om ontwikkelingsachterstand, epilepsie, gedragsstoornissen en lage bloedsuikers maar soms ook hele andere symptomen. Deze symptomen blijken nogal eens van voorbijgaande aard te zijn en soms verklaard te kunnen worden door een andere oorzaak. Vrijwel alle familieleden van patiënten die eveneens SCADD bleken te hebben hadden helemaal geen symptomen. Daarnaast kwam in hielprikscreening studies naar voren dat vrijwel alle kinderen met SCADD uit de hielprikscreening in de USA en Australië eveneens geen symptomen ontwikkelden. Bovendien bleken de symptomen die bij SCADD gezien worden ook de meest voorkomende symptomen te zijn bij kinderen waarbij stofwisselingsonderzoek wordt aangevraagd, wat suggereert dat toeval een rol speelt.

Op basis van deze resultaten wordt geconcludeerd dat SCADD zeer waarschijnlijk helemaal niet als stofwisselingsziekte moet worden beschouwd, maar als een veel voorkomende niet ziekmakende variant van de stofwisseling. Deze stofwisselingsvariant wordt dan uitsluitend gekenmerkt door een verhoogde concentratie aan bepaalde stofwisselingsproducten: C4-Carnitine in bloed en ethylmalonzuur in urine. De studieresultaten laten zien dat de hoogte hiervan is gerelateerd aan de ernst van de afwijking in het gen dat de informatie voor het SCAD enzym bevat. Maar ook dat de beschikbaarheid van flavine adenine dinucleotide, een vitamine B product dat nodig is voor de werking van het SCAD enzym en condities zoals vasten hierbij van invloed zijn. Het beschouwen van SCADD als een stofwisselingsvariant impliceert dat SCADD niet in hielprikscreening programma’s zou moeten worden opgenomen. Mede op grond van de beschreven studieresultaten is SCADD inmiddels verwijderd uit hielprikscreening programma’s in Australië en zal hopelijk ook hiertoe worden besloten in de USA.

In het geval van een verhoogd ethylmalonzuur en/of C4-Carnitine bij patiënten met symptomen dienen bepaalde stoornissen, die wel klinisch relevant zijn en eveneens gegaan met een verhoogde concentratie van deze stofwisselingsproducten, uitgesloten te worden. Als tijdens dit proces de diagnose SCADD wordt gesteld, zal aan patiënten en/of ouders moeten worden uitgelegd dat SCADD beschouwd dient te worden als een stofwisselingsvariant en dat verder aanvullend onderzoek naar mogelijke oorzaken van de symptomen uitgevoerd zal moeten worden.
Dankwoord
Dankwoord

Geweldig om de gelegenheid te krijgen iedereen die de afgelopen jaren heeft bijgedragen aan de totstandkoming van dit proefschrift te bedanken!

De patiënten en hun familieleden:
Zonder jullie medewerking hadden de in dit proefschrift beschreven studies niet plaats kunnen vinden. Heel veel dank daarvoor! Wat betreft de SCADD patiënten en hun ouders: Het was enorm stimulerend hoe gemotiveerd jullie waren bij het uitvoeren van vastentesten (hou het maar eens vol om een hele dag niet eten), vetbelastingstesten (smerige zonnebloemolie achterover slaan) en 60 minuten fietsen (en dat soms wel 4 keer!). Daarnaast leverden broertjes, zusjes en ouders zonder problemen buisjes bloed en potjes urine in en ondergingen soms zelfs zelf een test. Wat betreft de epilepsie patiënten en hun ouders: geweldig dat jullie besloten mee te doen aan onze studie en hiervoor tijd vrij maakten.

Mijn promotoren en co-promotor:
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elke mutatie in onze artikelen vermeld volgens de juiste nomenclatuur (de Denen bakken er niets van!). Heel veel dank!

De leden van mijn promotiecommissie:
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