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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Key words
- short-chain acyl-CoA dehydrogenase deficiency
- epilepsy
- clinical relevance

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 these variants in combination with a rare *ACADS* mutation, in the Netherlands predominantly the c.1058C>T. Epilepsy in childhood often remains unexplained and patients with epilepsy related to SCADD may remain undiagnosed because studies for SCADD are often not performed. To test this hypothesis and to further estimate the extent of the Dutch SCADD population, we performed a study on blood spot samples in 131 paediatric patients with epilepsy and 909 anonymous newborns and investigated the presence of the 2 common *ACADS* variants and the rare c.1058C>T mutation. Overall, the 2 common *ACADS* variants and the rare 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. A birth prevalence of SCADD with a mutation/variant genotype in the Netherlands as high as >1:1 000 was calculated. This is in contrast with the low number of patients diagnosed clinically and supports the hypothesis that SCADD is clinically irrelevant. Furthermore our study does not support an association between SCADD and epilepsy.

Abbreviations

ACADS	SCAD-encoding gene
C4-C	butyrylcarnitine
C4-CoA	butyryl-CoA
EMA	ethylmalonic acid
RIVM	National Institute for Public Health and the Environment
SCAD	short-chain acyl-CoA dehydrogenase
SCADD	short-chain acyl-CoA dehydrogenase deficiency

Introduction

Short-chain acyl-CoA dehydrogenase (SCAD) is the first enzyme of the short-chain β-oxidation pathway catalysing dehydrogenation of short-chain fatty acids. SCAD deficiency (SCADD) is a newly recognised autosomal recessive inborn error of fatty acid oxidation that has been associated with a remarkable variety of clinical symptoms. SCADD is probably one of the more common inborn errors of metabolism [23].

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.

The diagnosis of SCADD is usually confirmed by DNA analyses [23]. The majority of SCADD patients are homozygotes or compound heterozygotes for 2 common SCAD-encoding gene (*ACADS)* variants, i.e., c.625G>A and c.511C>T, or for these common *ACADS* variants in combination with a rare *ACADS* mutation [20,22–24]. Homozygosity for either of the common *ACADS* variants has been found in the general population with a remarkably high prevalence: approximately 0.3% for the common c.511C>T and 5.5% for the common c.625G>A variant [17,18]; and this *ACADS* genotype has been considered to confer susceptibility to the development of clinical

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disease [5,9,11]. More than 70 different *ACADS* mutations have been reported so far [5,10,11,14–16,19–21,23,24]. The majority of Dutch patients are compound heterozygous for the common c.625G>A variant and one specific rare mutation, c.1058C>T [23] located on the c.625G and c.511C *ACADS* alleles. Overall an *ACADS* genotype consisting of this specific rare mutation and/or common *ACADS* variants was present in 61% of Dutch SCADD patients [23].

Most of the reported SCADD patients were initially investigated because of neurological symptoms and/or hypoglycaemia [5,20,22–24]. In our previous study on 31 Dutch SCADD patients [23], developmental delay was reported in 52% and epilepsy in 35% of patients. Behavioural disorders and hypoglycaemia 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 were identified that explained the symptoms initially leading to metabolic studies.

Epilepsy is a frequent finding in childhood. Studies from Europe and North America report rates from 3.6 to 6.5/1000 children [1–3,6,8,12]. Diagnostic studies fail to establish the cause of epilepsy in up to 30% of the patients [13]. However, in patients with idiopathic childhood epilepsy, studies for inborn errors of metabolism such as SCADD, as a potential cause are often not performed. Furthermore, the facts that epilepsy is a frequent symptom in SCADD and that the prevalence of SCADD is much higher than the number of patients detected by metabolic studies [23], suggest that patients with epilepsy related to SCADD may remain undiagnosed.

To test this hypothesis we tested a group of paediatric patients with epilepsy for the presence of SCADD. We aimed to gain knowledge regarding the need for SCADD studies in epilepsy patients. In addition, we aimed to further estimate the extent of SCADD individuals in the Netherlands by establishing the frequencies of the common c.511C>T *ACADS* variant and the rare c.1058C>T *ACADS* mutation in the Dutch population.

Patients and Methods

Patients and material

We performed a study involving 131 paediatric patients with epilepsy and 909 anonymous newborns. Blood spots for the reference group were obtained from the RIVM (National Institute for Public Health and the Environment, the Dutch Institute involved in neonatal screening). The sample of newborn screening cards used was proportional to the number of live births in each of 14 screening districts, ensuring adequate demographic representation of the Dutch population. Dutch newborn screening cards are not released for any reason except newborn screening testing during the first 5 years. Consequently, the screening cards used in this study were more than 5 years old. Newborn screening cards were stored at 4 °C and they were analysed anonymously.

The study was performed in a paediatric patient group with patients aged 16 years or younger at the time of participation. Because all patients in the Dutch SCADD patients group were <8 years old when epilepsy symptoms started, we restricted the epilepsy group to those with onset of epilepsy at <8 years. The exclusion criteria included a known or suspected cause of the epilepsy and any previously performed metabolic studies (including SCADD investigations).

Patients with epilepsy were recruited via paediatricians and neurologists within the Academic Medical Center Amsterdam, and 3 Dutch epilepsy centres. Patient and symptom characteristics were obtained using questionnaires answered by parents or legal representatives.

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

DNA PCR-RFLP analysis

DNA was extracted from blood spot samples using Chelex 100 (BioRad) essentially as previously described [7,25], but with the following modifications. Blood spot samples (6 mm diameter) were washed overnight at 4 °C with 1 mL sterile water. The next day, the supernatant was discarded, 200 µL of Chelex (50 g/L, pH 10.6) were added and the sample was incubated at 56 °C for 30 min. Subsequently, the samples were mixed for 10 s, centrifuged (3 min at 10 000 × g), incubated for 8 min at 96 °C, mixed again for 10 s and centrifuged (3 min at 10 000 × g). From this mixture, 14.5 µL of supernatant were used in a 25-µL PCR reaction.

The extracted DNA was subjected to PCR-RFLP analysis to determine the presence of the common c.625G>A and c.511C>T *ACADS* variants and the rare c.1058C>T *ACADS* mutation. Concerning the common c.625G>A variant, a RFLP assay was used as previously described [17]. To identify the common c.511C>T variant and the rare c.1058C>T mutation, new PCR-RFLP assays were developed.

The common c.511C>T variant creates an *Mva*I restriction site. Exon 5 contains an additional *Mva*I site 42 bp downstream that is used as an internal control for restriction. The upstream endogenous *Mva*I 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 *ACADS* gene is amplified from 14.5 µL extracted DNA in a 25 µL PCR reaction as previously described [7], except for the use of 1.25 mM $MgCl_2$. The following primers were used: forward 5′-tgtaaaacgacggccagt CGT GCG CTG AGC **A**CT GGG TCT-3′, reverse 5V-caggaaacagctatgacc TCG AAG CCT CCC AGG CAT TGG TGA-3′. Similar to the reverse primer for the PCR-RFLP analysis of the common c.625G>A variant, the forward and reverse primers for determining the common c.511C>T variant have an M13-rev and -21M13 extension (lower case letters), that can be used for sequence analysis.

The rare c.1058C>T mutation creates an *Eco*130I (*Sty*I) restriction site. Exon 9 contains an additional *Eco*130I (*Sty*I) site 18 bp upstream that is used as an internal control for restriction. For this PCR-RFLP analysis, exon 9 of the *ACADS* gene is amplified from 14.5 µL extracted DNA in a 25 µL PCR reaction as previously described [17] using 1.0 mM $MgCl_2$ and using 5′-tgtaaaacgacggccagt GGG AAG GCT CTG ACT GTA CC-3′ as forward primer and 5′-caggaaacagctatgacc CAG GAT CTG GAT GGC CTG AG-3′ as reverse primer. These primers also have an M13-rev and -21M13 extension (lower case letters).

For DNA amplification a PTC 100 thermocycler was programmed as follows: denaturation for 2 min at 96 °C, followed by 5 cycles of 30 s at 96 °C, 30 s at 55 °C and 30 s at 72 °C; this is followed by 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.

Table 1 Frequency of *ACADS* genotypes in study populations and in the epilepsy group.

	Nagan study [18] (n = 694)	Previous study [17] (n = 1 036)	Current study (n = 909)		Epilepsy group (n = 131)
			Observed	Expected*	
511C/C			91.0 %	91.1 %	90.8 %
511T/C	5.6 %		8.9 %	8.7 %	9.2 %
511T/T	0.3 %		0.1 %	0.2 %	0 %
625G/G			59.7 %	59.7 %	55.7 %
625A/G	31.8 %	31.3 %	35 %	35 %	36.6 %
625A/A	5.6 %	5.5 %	5.3 %	5.3 %	7.6 %
1058C/C			99.3 %	99.4 %	99.2 %
1058T/C			0.7 %	0.6 %	0.8 %
combinations					
1058T/C + 625A/G			0.1 %	0.07 %	0 %
625A/G + 511T/C	1.0 %		2 %	1.04 %	1.5 %
511T/T, 625A/A, 1058T/C + 625A/G, 625A/G + 511T/C			7.5 %**	6.6 %	9.2 %**

* Calculated from the observed allele frequencies assuming Hardy-Weinberg equilibrium

** Not significantly different (OR 1.3, 95 % CI 0.7–2.4)

RFLP c.625G>A: To 25 µL of PCR product, 2.9 µL Buffer M and 5 U *Dde*I (Roche) were added, and these additions were followed by overnight incubation at 37 °C. In the presence of the c.625G variant, 2 restriction fragments (208 and 18 bp) are observed; in the presence of the c.625A variant, 3 fragments (180, 28, and 18 bp) are observed. Thus, heterozygosity for the common c.625G > A variant will yield fragments of 208, 180, 28, and 18 bp. The undigested PCR fragment is 226 bp.

RFLP c.511C>T: To 25 µL of PCR product 2.9 µL Buffer R and 10U *Mva*I (Fermentas) were added, and these additions were followed by overnight incubation at 37 °C. The undigested PCR fragment of the c.511C>T variant is 175 bp. The digested PCR product shows 2 fragments (130 and 45 bp) in a non-carrier of the common c.511C>T variant and fragments of 130, 88, 45, and 42 bp when the common c.511C>T variant is present in one of the alleles.

RFLP 1058C>T: To 25 µL of PCR product 2.9 µL Buffer O and 10 U *Eco*130I (*Sty*I) (Fermentas) were added, and these conditions were followed by overnight incubation at 37 °C. The undigested PCR fragment of the rare c.1058C>T. mutation is 238 bp. The digested PCR product shows 2 fragments (179 and 59 bp) in the wild-type and and 4 fragments (179, 161, 59, and 18 bp) when the c.1058C>T mutation is present in heterozygous form. Restriction fragments were analysed on a 2 % (w/v) agarose gel stained using ethidium bromide. Before loading 1/10 volume loading dye (150 g/L Ficoll 400, 1.5 g/L orange G, and 0.14 g/L xylene cyanol FF) was added to samples. A 100 bp ladder (Invitrogen) was used to estimate fragment sizes.

Statistical analyses

SPSS (SPSS 12.0.1), Graphpad Prism 3.0 and CIA (version 1, 1989) software programmes were used to analyse the data from the DNA studies.

The sample size necessary for this study was calculated based on previous studies that estimate that 6 % of normal (reference) individuals have *ACADS* genotypes that are either homozygous or compound heterozygous for the common *ACADS* variants and/or the rare c.1058C>T mutation [17, 18, 23]. A 3-fold higher frequency, i.e., 18 % in the patient group was supposed to indicate an association between these specific *ACADS* genotypes and epilepsy. Using a 2-tailed test with $\alpha = 0.05$ and power = 80 %, we estimated that a sample size of at least 65 patients and 260 reference subjects was required.

To estimate the association between the *ACADS* genotypes and epilepsy, the odds ratio was calculated.

Results

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

The distribution of genotypes determined for the common c.511C>T variant clearly favoured the wild-type allele. In 81 of the 909 individuals (8.9 %) one copy of the common c.511C>T variant was identified and one individual (0.1 %) was homozygous for this variant (▸ **Table 1**). Homozygosity and heterozygosity for the common c.625G > A variant were far more prevalent, with percentages of 5.3 and 35 %, respectively. In 18 of the 909 individuals (2 %) compound heterozygosity for the 2 different common variants was established. In 6 of the 909 individuals (0.7 %) one copy of the rare c.1058C>T mutation was identified. One of these 6 individuals also carried one copy of the common c.625G > A gene variant. Based on the observed allele frequencies of 4.6, 22.8 and 0.3 % for the common c.511C>T and c.625G>A gene variants and the rare c.1058C>T mutation, respectively, the expected genotype frequencies were calculated, assuming Hardy-Weinberg equilibrium (▸ **Table 1**).

Overall, the common c.511C>T or c.625G>A gene variant or the rare c.1058C>T mutation were detected in either homozygous or compound heterozygous forms along with 1 of the other 2 in 68/909 (7.5 %, 95 % CI 5.9–9.4 %) subjects from the Dutch population.

Demographic data of the epilepsy study group

Data from 131 epilepsy patients (66 girls and 85 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. Normal development was confirmed in 92/131 (70 %) patients and 39/131 (30 %) patients had delayed cognitive and/or motor function.

Incidence of the common 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 common c.511C>T variant was identified, but none of the epilepsy patients were homozygous for this variant (▶ **Table 1**). The common c.625G>A variant was identified in heterozygous form in 48 patients (36.1 %) and in homozygous form in 10 (7.5 %) patients. One of the patients (0.8 %) carried one copy of the rare c.1058C>T mutation. Overall, the common c.511C>T variant, the common c.625G>A variant or the rare c.1058C>T mutation were detected in either homozygous or compound heterozygous forms along with 1 of the other 2 in 12/131 (9.2 %, 95 % CI 4.8–15.5 %) epilepsy patients. This was comparable to the reference group (7.5 %, ▶ **Table 1**). Within the epilepsy group, the combined *ACADS* genotypes were established in 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.

Discussion

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The current study is the first to investigate the prevalence of the common c.511C>T *ACADS* variant and the rare c.1058C>T *ACADS* mutation in the Netherlands. In addition, it is the first study to examine a potential association between SCADD and one of its most frequently associated symptoms: epilepsy. This study included 131 patients and 909 reference subjects, resulting in a power of >98 % to detect a difference of at least 12 % between the reference and the patient groups.

Homozygosity and heterozygosity for the common c.511C>T *ACADS* variant in the Dutch population (0.1 and 8.9 %, respectively) were comparable to the percentages reported for the USA population (▶ **Table 1**) [18]. The prevalence of the most common *ACADS* variant, c.625G>A, in the Netherlands, has been investigated previously [17], and the current study provides similar results (5.3 and 35 %, respectively, ▶ **Table 1**). Because those results are also comparable to numbers reported for the USA population, it appears that both common *ACADS* variants, which have been considered to confer susceptibility to the development of clinical SCADD, are represented proportionally throughout the Western world. If these common *ACADS* variants would indeed confer susceptibility for clinical SCADD, this would imply every 1:14 Western newborns to be at risk for "SCADD symptoms". In the Netherlands, which has an annual birth rate of 180 000 [4], this corresponds to approximately 12 500 newborns each year.

In addition we investigated the prevalence of the rare c.1058C>T mutation, which is thought to represent a Dutch founder mutation [23]. The results of the current study provide evidence for a founder mutation because 0.7 % percent of the reference group was identified with one c.1058C>T copy, all patients carrying the rare c.1058C>T mutation were of Dutch ancestry, and as this rare mutation has not been reported in patients without Dutch ancestors [23]. Based on the established frequencies for this rare Dutch mutation and the common *ACADS* variants, a birth prevalence as high as 1:1 000 for an *ACADS* genotype compound heterozygous for an common *ACADS* variant plus the rare c.1058C>T mutation, can be calculated. This *ACADS* genotype has been associated with the biochemical features (increased C4-C and/or EMA) of SCADD in all previously reported cases [23]. For the Netherlands this implies that, in addition to approximately 12 500 newborns with supposed susceptibility for clinical 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 1:1 000, which is significantly higher than our previous estimate of 1:3 300 [23]. This is in strong contrast with the fewer than 40 patients with SCADD who have been diagnosed in the Netherlands in recent decades. Failure to diagnose SCADD may be explained if SCADD often presents with clinical signs and symptoms that do not lead to metabolic studies of urine or plasma for increased EMA and/or C4-C concentrations. Because epilepsy appears to be a common clinical symptom in SCADD, and uncomplicated epilepsy in childhood is usually not followed by diagnostic tests for metabolic diseases, a high prevalence of SCADD in patients with unexplained epilepsy may, in part, explain the high number of unrecognised SCADD patients. However, failure to diagnose SCADD may also be explained if SCADD only represents a biochemical condition, not leading to any symptoms.

We therefore investigated the presence of the most common *ACADS* genotypes (representing 61 % percent of *ACADS* genotypes in the Dutch SCADD patient group) in paediatric patients with epilepsy of unknown origin as well as in reference subjects. However, our study failed to demonstrate an association between SCADD, defined as homozygosity or compound heterozygosity for the common c.511C>T and c.625G>A variant and/or the Dutch rare c.1058C>T mutation and epilepsy of unknown origin in childhood.

A first limitation of our study is that we could not exclude an association between idiopathic childhood epilepsy and SCADD with rare mutations on both alleles. In order to do this very high numbers of patients and reference subjects would have been needed, due to the fact that homozygosity or compound heterozygosity for these mutations is rare. A second limitation is that we cannot rule out an association of epilepsy with the studied *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 only 2.5 % and therefore not very likely.

In conclusion, our study shows that the prevalence of SCADD in the Netherlands is at least 1/1 000 newborns. Because this is in contrast with the low number of patients diagnosed clinically, it contributes to the hypothesis that SCADD has no clinical relevance. Our study does not provide evidence for an association between SCADD based on the most common *ACADS* genotypes and epilepsy. This suggests that SCADD is not an important risk factor for the development of epilepsy and metabolic investigations aimed to diagnose SCADD for childhood epilepsy of unknown origin cannot be recommended.

Acknowledgements

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