Short-chain acyl-CoA dehydrogenase deficiency
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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 DdeI. 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 CGC CTC TCC TTT CTG -3', Reverse 5'- caggaaacagctatgacc TGA GCA CCA TGG CTA TCT TGA AGC -3'. The reverse primer introduces an additional DdeI 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 DdeI 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 DdeI (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.\textsuperscript{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.\textsuperscript{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 $C_4$ and free ($C_0$-) carnitine levels ($\mu$mol/L) and the subsequent $C_4:C_0$ carnitine ratios in newborn screening cards homozygous, heterozygous, and of non-carriers for the c.625G>A ACADS variant.

<table>
<thead>
<tr>
<th></th>
<th>Homozygous</th>
<th>Heterozygous</th>
<th>Non-carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean $C_4$-Carnitine ($\mu$mol/L)</td>
<td>0.16</td>
<td>0.13</td>
<td>0.15</td>
</tr>
<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 $C_0$-Carnitine ($\mu$mol/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 $C_4:C_0$-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 $C_4$-Carnitine: 0.14-0.87 $\mu$mol/L. Normal range $C_0$-Carnitine: 24.7-66.6 $\mu$mol/L.

Figure. $C_4$-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.\(^{13-15}\) 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.\(^9\) 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.


