Females heterozygous for creatine transporter deficiency
Clinical features and X-inactivation in females heterozygous for creatine transporter deficiency

van de Kamp JM, Mancini GM, Pouwels PJW, Betsalel OT, van Dooren SJM, de Koning I, Steenweg ME, Jakobs C, van der Knaap MS, Salomons GS

ABSTRACT

The creatine transporter deficiency is an X-linked cause of intellectual disability. We investigated the clinical features and pattern of X-inactivation in a Dutch cohort of eight female heterozygotes. We show that symptoms of the creatine transporter deficiency (intellectual disability, learning difficulties, constipation) can be present in female heterozygotes. We further show that the diagnosis in females is not straightforward: (1) The creatine/creatinine ratio in urine was elevated only in three out of eight females. (2) Although as a group the females had a significantly decreased cerebral creatine concentration, individual females had creatine concentrations overlapping with normal controls. (3) Skewed X-inactivation was found in the cultured fibroblasts, in favor of either the mutated or the wild-type allele, leading to either deficient or normal results in the creatine uptake studies in fibroblasts. Thus, screening by these tests is unreliable for the diagnosis. In addition, we found no consistent skewing of the X-inactivation in peripheral tissues indicating that there is no selection against the creatine transporter deficiency. We conclude that testing for creatine transporter deficiency should be considered in females with (mild) intellectual disability. Screening by DNA analysis of the SLC6A8 gene is recommended.
INTRODUCTION

Creatine transporter deficiency is an X-linked cause of intellectual disability with a prevalence of 0.3%-3.5% in males with intellectual disability. The first male patient with creatine transporter deficiency was described in 2001. Since then several male patients have been reported. Patients present with intellectual disability, severe speech delay, behavior disturbances, and epilepsy. X-linked creatine transporter deficiency forms together with the autosomal recessive creatine biosynthesis defects arginine:glycine amidinotransferase (AGAT) deficiency and guanidinoacetate methyltransferase (GAMT) deficiency, the group of cerebral creatine deficiency syndromes, which are all characterized by almost complete absence of the creatine peak in $^1$H-magnetic resonance spectroscopy (MRS) of the brain. An increased creatine/creatinine ratio in urine is used as marker for the diagnosis of creatine transporter deficiency in male patients, although this test has a high rate of false positive results. DNA analysis of the SLC6A8 gene and/or creatine uptake studies in cultured fibroblasts are used to confirm the diagnosis.

Because creatine transporter deficiency is an X-linked condition, the phenotype in females is expected to be influenced by the X-inactivation pattern. Learning difficulties or mild intellectual disability have been mentioned in several heterozygous females in the reported creatine transporter deficiency families but with few clinical details. We report the systematic study of clinical features and X-inactivation pattern in heterozygous females in eight Dutch creatine transporter deficiency families to answer the following questions: (1) Do females who are heterozygous for creatine transporter deficiency present with symptoms? (2) How do we diagnose heterozygous females? And, (3) what is the X-inactivation pattern in heterozygous females and is there a correlation with the phenotype?

METHODS

Subjects

Twelve index boys with creatine transporter deficiency were diagnosed in the Netherlands till 2007. Nine out of 11 mothers tested for the mutation were found to be (non-mosaic) carriers and one mother had a low-level somatic mosaicism. In addition, two sisters of index boys were found to be heterozygous. All heterozygous females were diagnosed by DNA analysis.
Chapter 3     Female heterozygotes

All 11 non-mosaic heterozygous females were invited to participate in the study of which three declined. Eight heterozygous females, aged 32 to 77 years (mean age 47 years), from eight families were included in the study. All participants gave informed consent. The study was approved by the ethics committee of the VU University Medical Center, Amsterdam, the Netherlands.

Clinical evaluation
All heterozygous females were seen by the authors (J.K. and G.M.). A medical and family history was taken and physical and neurologic examination was performed.

Neuropsychological assessment
To estimate general intelligence, we used the short version of the Groninger Intelligence Test 2 (GIT-2), a Dutch intelligence test. Education was categorized according to the system of Verhage.

Biochemical analysis
Guanidinoacetate (GAA) and creatine (Cr) were measured in plasma and urine using stable isotope dilution gas chromatography-mass spectroscopy according to Almeida et al. Creatinine (Crn) in urine was measured with the Jaffé method.

Magnetic resonance imaging and MRS
Magnetic resonance examinations were performed at 1.5T (Siemens Vision, Erlangen, Germany) using a standard circularly polarized head coil.

For MRS, volumes-of-interest (VOIs) in parietal cortex (10-12 ml), parietal white matter (5 ml), and cerebellar vermis (8 ml) were positioned on axial and coronal T2-weighted images and on 3D T1-weighted sagital images. In each VOI, a fully relaxed, short echo time STEAM spectrum (repetition time/echo time/mixing time = 6000/20/10 ms; 64 acquisitions) was obtained and spectra were quantified using LCModel. In this study, concentrations of total NAA (sum of N-acetylaspartate and N-acetylaspartyl glutamate), total Cr (sum of creatine and phosphocreatine), Cho (choline-containing compounds), and Ins (myo-Inositol), were considered, and expressed in mmol/l VOI (mM). Metabolite concentrations were compared with data from healthy controls, obtained from a local database of 29 subjects (mean age 36, range 25 – 62 years) with one to three spectra per subject. Statistical comparison was performed with an unpaired t-test.
Clinical features and X-inactivation in female heterozygotes

**Creatine uptake assay**
The creatine uptake assay in cultured skin fibroblasts was performed according to Rosenberg et al.\textsuperscript{24} Creatine uptake was measured after incubation with 25μM creatine. The measured intracellular creatine concentration is expressed in picomol creatine per microgram total protein. The incubations were performed in triplicate.

**X-inactivation studies**
The X-inactivation pattern was determined by polymer chain reaction (PCR) analysis of a polymorphic (CAG)\textsubscript{n} repeat in the first exon of the androgen receptor (AR) gene with and without digestion of the DNA with the methylation-sensitive enzyme \textit{HhaI}.\textsuperscript{31} All samples were analysed in triplicate. A male control was included in each run. PCR products were separated on an ABI 3130xl automated sequencer (Applied Biosystems) and peak areas of both alleles were measured, ignoring any stutter peaks, with GeneScan software (Applied Biosystems). To compensate for preferential amplification of one of the alleles, the peak areas of the digested and undigested samples were compared using the following calculation: \( \% \text{ inactive A1} = 100 \times \frac{(A1^+/A1^-)/[(A1^+/A1^-) + (A2^+/A2^-)]}{(A1^+/A1^-) + (A2^+/A2^-)} \) where A1 and A2 represent the smaller allele and the larger allele and + and − represent the digested and undigested samples respectively.\textsuperscript{32} The X-inactivation pattern was determined in DNA obtained from peripheral blood leukocytes, hair roots, saliva and cultured skin fibroblasts (passages 4-8).

**Analysis of the SLC6A8 gene**
In all families genomic sequence analysis of the \textit{SLC6A8} gene was performed according to Rosenberg et al.\textsuperscript{1}

**Statistical analysis**
The correlation between intelligence quotient (IQ) scores and respectively cerebral creatine concentrations and creatine/creatinine ratio in urine was studied using the Pearson correlation coefficient.

**RESULTS**

**Medical history**
The medical history is summarized in Table 1. Intellectual disability was evident in one female (individual 1). One other female required special education and three females failed
**Table 1.** Clinical features of heterozygous females

<table>
<thead>
<tr>
<th>Individual</th>
<th>Age (years)</th>
<th>Development</th>
<th>Learning difficulties</th>
<th>Other symptoms</th>
<th>Neurological examination</th>
<th>Verhage$^a$</th>
<th>IQ 95% CI</th>
<th>Urine Cr/Cr (µmol/L)</th>
<th>Plasma Cr (µmol/L)</th>
<th>tCr brain (% normal mean)</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42</td>
<td>Mild MD/SD, PT, ST</td>
<td>Special education sheltered workplace</td>
<td>Possibly seizures at 12-14 years</td>
<td>Mild cerebellar symptoms</td>
<td>3</td>
<td>41-55</td>
<td>0.679</td>
<td>91</td>
<td>66</td>
<td>c.1495+5G&gt;C</td>
</tr>
<tr>
<td>2</td>
<td>65</td>
<td>Normal</td>
<td>Failed year in primary school</td>
<td>Severe constipation</td>
<td>Normal</td>
<td>4</td>
<td>66-80</td>
<td>0.337</td>
<td>44</td>
<td>70</td>
<td>c.1011C&gt;G p.(Cys337Trp)</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>Normal</td>
<td>No</td>
<td>Breast cancer at 38 years</td>
<td>Normal</td>
<td>5</td>
<td>65-80</td>
<td>0.098</td>
<td>44</td>
<td>87</td>
<td>c.1631C&gt;T p.(Pro544Leu)</td>
</tr>
<tr>
<td>4</td>
<td>41</td>
<td>Normal, ST</td>
<td>Special education</td>
<td>EEG for unknown reason at 10 years Breast cancer at 41 years</td>
<td>Normal</td>
<td>4</td>
<td>65-79</td>
<td>0.384</td>
<td>62</td>
<td>78</td>
<td>c.570_571del p.(Ala191GlnfsX10)</td>
</tr>
<tr>
<td>5</td>
<td>43</td>
<td>Normal</td>
<td>Failed year in primary school</td>
<td>Normal</td>
<td>4</td>
<td>65-79</td>
<td>0.059</td>
<td>38</td>
<td>87</td>
<td>c.428_430del p.(Tyr143del)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>32</td>
<td>Walking at 2 years</td>
<td>No</td>
<td>Irritable bowel syndrome with constipation</td>
<td>Mild cerebellar symptoms</td>
<td>5</td>
<td>78-92</td>
<td>0.065</td>
<td>30</td>
<td>83</td>
<td>c.92delC p.(Pro31ArgfsX66)</td>
</tr>
<tr>
<td>7</td>
<td>35</td>
<td>Normal</td>
<td>No</td>
<td></td>
<td>Normal</td>
<td>5</td>
<td>89-103</td>
<td>0.057</td>
<td>35</td>
<td>82</td>
<td>c.778-300_1764del</td>
</tr>
<tr>
<td>Individual</td>
<td>Age</td>
<td>Development</td>
<td>Learning difficulties</td>
<td>Other symptoms</td>
<td>Neurological examination</td>
<td>Verhage&lt;sup&gt;a&lt;/sup&gt;</td>
<td>IQ</td>
<td>Urine Cr/Crn</td>
<td>Plasma Cr</td>
<td>tCr brain (% normal mean)</td>
<td>Mutation</td>
</tr>
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<tr>
<td>8</td>
<td>77</td>
<td>Normal</td>
<td>Failed year in primary school</td>
<td>Normal</td>
<td>Normal</td>
<td>3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53-67</td>
<td>0.051</td>
<td>31</td>
<td>65</td>
<td>c.1299_1309del p.(Pro434LeufsX27)</td>
</tr>
</tbody>
</table>

Normal controls: 0.011-0.244<sup>c</sup> 6-50<sup>c</sup>

Affected boys: 1.4-5.5<sup>c</sup>

<sup>a</sup> Verhage scoring system<sup>29</sup>: 1, no completed primary education; 2, completed primary education; 3, some secondary education; 4, completed secondary education in a preparatory vocational education (vbo); 5, completed secondary education in a general continued education (mavo) and/or completed tertiary education in vocational education (mbo); 6, completed secondary education in a higher general secondary education (havo) or pre-university education (vwo) and/or completed tertiary education in a higher professional education (hbo); 7, completed university education.

<sup>b</sup> Secondary school not finished because of World War II.

<sup>c</sup> From Almeida et al.<sup>21</sup>

CI, confidence interval; Cr, creatine; Crn, creatinine; EEG, electroencephalogram; IQ, intelligence quotient; MD, motor delay; PT, physical therapy; SD, speech delay; ST, speech therapy.
a year at elementary school. Most females had an educational level scored according to Verhage of 4 or 5, which is average.

One female (individual 2) had severe constipation from the age of 55 years, for which she had a sacral nerve stimulator implanted at the age of 62 years. She also had a period of constipation for which she was admitted to hospital at the age of 20 years.

**Family history**

Of the eight participating females, six were mothers of one or more affected sons. The clinical features of four affected sons of two mothers were published previously.

The two other participating females were sisters of affected male patients. The clinical features of the two brothers of one of these females have been published previously.

**Physical examination**

No consistent evident dysmorphisms were detected. Body mass index (BMI) varied from 17 to 38 (mean 27), height varied from -2.3 to +2.7 standard deviation (SD) (mean -0.7 SD) and head circumference from -0.5 to +0.7 SD (mean -0.1 SD). Neurological examination (Table 1) revealed very mild cerebellar symptoms in two females with mild dysdiadochokinesis in the rapid alternating movements of the hands, mild dysmetria in the point-to-point tests (finger-nose tip and heel-knee tests), mild dysarthria (inability to pronounce “pataka”), nystagmus on lateral gaze and slight gait ataxia at heel-to-toe walking. One of them also had a unilateral tic of the mouth. Muscle tone and strength, deep tendon reflexes and sensory tests were normal in all females.

**Neuropsychological assessment**

IQ scores on the shortened GIT-2 varied from 48 to 96 with a 95% confidence interval (CI) of ± 7 (Table 1). Two females (1 and 8) had IQ scores in the intellectual disability range (IQ < 70) and four in the range of borderline intellectual functioning (IQ 70-85).

**Biochemical analysis**

GAA in urine and plasma was in the normal range in all females. The creatine/creatinine ratio in urine was mildly elevated in 3 females (Table 1). Two females also had a mildly elevated creatine in plasma.
Magnetic resonance imaging and MRS

No abnormalities were detected on magnetic resonance imaging. As a group, the heterozygous females had significantly decreased total creatine concentrations in cortex ($P=0.002$), white matter ($P<0.0001$) and cerebellum ($P=0.0001$) compared to normal controls (Table 2). Yet, individual females had creatine levels overlapping with normal controls (Figure 1). Individual results are mentioned in Table 1 as percentage of normal (measured value/mean of controls $\times$ 100) averaged over the three regions. For the other metabolites no significant differences were observed between the heterozygous females and the controls, with the exception of a significantly increased total NAA in the cortex of the heterozygotes ($P=0.036$) but not in white matter and cerebellum.

![Figure 1](image_url)

**Figure 1.** Total creatine concentration in the cortex of heterozygous females (black dots) plotted as a function of age, compared to normal controls (open dots) and affected male patients (black diamonds) (a) $^1$H-MRS STEAM spectrum in cortex of a normal control (b), a heterozygous female (c) and an affected male patient (d).

NAA, N-acetylaspartate; Cr, creatine and phosphocreatine; Cho, choline-containing compound; Ins, myo-inositol.
Table 2. Metabolite concentrations obtained from $^1$H-MRS (mean ± SD, in mM) in cortex, white matter and cerebellum of heterozygous females versus controls.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Cortex</th>
<th>White matter</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heterozygotes (n=8)</td>
<td>Control (n=24)</td>
<td>Heterozygotes (n=8)</td>
<td>Control (n=15)</td>
</tr>
<tr>
<td>tCr</td>
<td>4.5 ± 0.8</td>
<td>5.8 ± 0.4</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>tNA</td>
<td>8.5 ± 0.7</td>
<td>7.8 ± 0.5</td>
<td>7.9 ± 1.2</td>
</tr>
<tr>
<td>Cho</td>
<td>0.89 ± 0.10</td>
<td>0.96 ± 0.11</td>
<td>1.43 ± 0.18</td>
</tr>
<tr>
<td>Ins</td>
<td>3.8 ± 0.5</td>
<td>3.8 ± 0.4</td>
<td>3.7 ± 0.5</td>
</tr>
</tbody>
</table>

Cho, choline-containing compounds; Ins, myo-inositol; ns, not significant; tCr, total creatine; tNA, total N-acetylaspartate.
Clinical features and X-inactivation in female heterozygotes

**Creatine uptake assay**

Creatine uptake assay in cultured skin fibroblasts was performed in seven out of the eight heterozygotes because the culture of skin fibroblasts failed in one female. Results were compared with uptake assays in twelve male SLC6A8-deficient patient fibroblasts and 13 normal control fibroblasts (Figure 2). Creatine uptake in cultured skin fibroblasts was in the normal range in four heterozygous females (individuals 4, 6, 7, and 8). In three females the uptake was somewhat above the deficient range (individuals 2, 3, and 5).

![Figure 2](image-url)  
**Figure 2.** Creatine uptake in skin fibroblasts of heterozygous females (white, numbered), normal controls (gray) and deficient controls (black) with incubations of 25 μM creatine. The values represent the mean ± SD of triplicate incubations of the heterozygous females and the mean ± range of 13 normal controls and 12 deficient controls.

**X-inactivation studies**

One heterozygous female (individual 8) was uninformative for the (CAG)$_n$ repeat polymorphism and the X-inactivation pattern could therefore not be determined. X-inactivation patterns of the other seven females are shown in Figure 3. In all, six females of whom X-inactivation studies in cultured skin fibroblasts were available, a severely skewed pattern was detected in the skin fibroblasts. Because the creatine uptake in the cultured skin fibroblasts was either in the normal range or slightly above the deficient range, it was possible to determine which (CAG)$_n$ repeat size associated with the wild-
type allele and the mutated allele because the most active allele in the fibroblasts with a normal uptake must be the wild-type allele and the most active allele in the deficient fibroblasts must be the mutated allele. Two females had a skewed X-inactivation pattern of 80:20 or more in blood leukocytes. In one (individual 7), this was in favor of the wild-type allele and she had the highest IQ score in this study. In the other (individual 5), the skewing was in favor of the mutated allele but she did not have a more pronounced abnormal phenotype.

![Figure 3. X-inactivation in blood leukocytes (b), saliva (s), hair roots (h), and cultured skin fibroblasts (f) in seven heterozygous females (1-7). The percentage of active wild-type allele is shown in black and of active mutated allele in gray. In individual 1 it is unknown which the wild-type allele is and which the mutated allele. This uncertainty is depicted with the gray striped area.](image)

**Analysis of the SLC6A8 gene**

All females included in this study were heterozygous for the mutation in the *SLC6A8* gene that had previously been found in related affected males (Table 1).

**Statistical analysis**

The correlation lines between IQ scores and respectively cerebral creatine concentrations and creatine/creatinine ratio in urine are shown in Figure 4. A Pearson correlation of respectively 0.65 (r squared = 0.43) and -0.64 (r squared = 0.40) was found. These correlations are however not significant.
Clinical features and X-inactivation in female heterozygotes

DISCUSSION

We detected symptoms of creatine transporter deficiency in female heterozygotes. Mild intellectual disability was evident in one heterozygote with developmental delay, learning difficulties requiring special education and an IQ score well below 70. IQ scores were < 70 (intelectual disability range) in one other female and between 70 and 85 (borderline intellectual functioning) in four other females, one of whom required special education. The IQ scores should however be interpreted with caution. Because of the Flynn effect (mean performance on IQ tests increases from one generation to the next), the norms for the GIT-IQ test have recently been updated (GIT-2) and higher IQ scores would have been found if the original GIT or other older IQ test would have been used. In some of the females, the IQ scores were lower than was expected based on educational level and clinical impression. Because only one of the females had an unaffected sister, as proven with mutation analysis, the IQ scores could not be compared with IQ scores of non-affected female siblings. Previous reports describe IQ scores of 67-99 in six heterozygous females. 12, 13, 15

One female possibly had seizures during puberty. Mild cerebellar symptoms were present in two females. Remarkable was the severe constipation from the age of 55 year in a 65-year-old female. Gastrointestinal problems including chronic constipation, megacolon, ulcer disease, ileus and bowel perforation, have been described in adult males with creatine transporter deficiency which include the two brothers of the heterozygous

Figure 4. Correlations between IQ scores and respectively total creatine concentrations in the brain and urinary creatine/creatinine ratio. Cr, creatine; crn, creatinine; GIT IQ, Groninger Intelligence Test Intelligence Quotient; tCr, total creatine
female with severe constipation in this study.\textsuperscript{15, 16} Severe constipation and ileus might be a complication of creatine transporter deficiency that develops later in life and can affect heterozygous females as well. Breast cancer was diagnosed at a relative young age in two heterozygous females. We could not relate this with the creatine transporter deficiency.

In this study, there is a selection bias for less severely affected, reproductively fit heterozygous females as six out of eight females were diagnosed because of an affected son. Therefore, our results can not be used without reservation to predict the chance of symptoms in heterozygous girls. However, our results support the assumption that creatine transporter deficiency can also be a cause of intellectual disability and learning difficulties in females. In fact, creatine transporter deficiency has been diagnosed in girls presenting with intellectual disability.

The diagnosis is probably often missed because we found that the diagnosis in females is not straightforward. Screening for an elevated creatine/creatinine ratio in urine is used to detect male patients but seems to be very unreliable in females. An elevated creatine/creatinine ratio was detected in only three out of the eight females in this study and the elevation was very mild. Creatine depletion on MRS of the brain is a hallmark of the disease in male creatine transporter deficiency patients. In all females, a relatively low cerebral creatine signal was found but there was overlap with normal controls. Therefore, a normal creatine/creatinine ratio in urine or a (low) normal MRS does not exclude the diagnosis in females. However, it is possible that more severely affected females more often have an elevated urinary creatine/creatinine ratio and a lower cerebral creatine concentration than mildly affected females and are therefore more easy to diagnose. We did indeed find a positive correlation between IQ scores and cerebral creatine concentrations and a negative correlation between IQ scores and urinary creatine/creatinine ratio. However these correlations were not significant probably due to the small sample size.

Creatine uptake studies in fibroblasts are used to confirm the diagnosis in males. Impaired creatine uptake was detected in fibroblasts of three out of seven females and could thus confirm the diagnosis but the other four had a normal uptake.

DNA analysis of the \textit{SLC6A8} gene (open reading frame 1.9 kb, 13 exons) is most likely the only reliable option for screening for creatine transporter deficiency in females presenting with (mild) intellectual disability. DNA testing for creatine transporter deficiency in currently not systematically included in the diagnostic workup of females with intellectual disability.

Some X-linked intellectual disability syndromes have been associated with skewed X-inactivation in blood cells indicating that there is selection against those cells in which
the mutation is located on the active X-chromosome.\textsuperscript{34, 35} We did not find consistent skewing in peripheral blood leukocytes, hairs, and saliva, indicating that there is no selection against cells with creatine transporter deficiency. In the absence of selection, the phenotype is expected to vary from normal to (severely) abnormal with the by chance variation of the X-inactivation from favorably to unfavorably skewed.\textsuperscript{36} This corresponds to our finding of symptoms in some of the females. In practice, X-inactivation analysis is usually performed on blood cells. We did not find a correlation between X-inactivation in blood cells and phenotype. This is not unexpected as the X-inactivation pattern in blood does not necessarily predict the pattern in the brain. Surprisingly, we did find 88-99% skewing in the cultured skin fibroblasts in all six females which might be due to clonal selection in the culturing. Further passages of fibroblast cultures showed further skewing (unpublished observations). The selection was however not directed at the creatine transporter mutation as we found skewing both in favor of the mutated allele as the wild-type allele. We hypothesize that the selection is directed at another, unrelated, factor. Likewise Plagnol et al. showed that culturing in lymfoblastoid cell lines often leads to mono- or pauciclonality.\textsuperscript{37} Importantly, this severe skewing in cultured fibroblasts, makes the study of creatine uptake in cultured fibroblasts unreliable for the diagnosis of creatine transporter deficiency in female heterozygotes.

In conclusion, this study shows that females who are heterozygous for creatine transporter deficiency can have symptoms of this condition. Testing for this condition should be considered in females with (mild) intellectual disability but screening based on urinary creatine/creatinine ratio is not reliable and screening with DNA analysis of the SLC6A8 gene is recommended.

**ACKNOWLEDGMENTS**

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