

Short Report

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

van de Kamp JM, Mancini GMS, Pouwels PJW, Betsalel OT, van Dooren SJM, de Koning I, Steenweg ME, Jakobs C, van der Knaap MS, Salomons GS. Clinical features and X-inactivation in females heterozygous for creatine transporter defect.

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The creatine transporter defect is an X-linked cause of mental retardation. 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 defect (mental retardation, learning difficulties, and constipation) can be present in female heterozygotes. We further show that the diagnosis in females is not straightforward: (i) The creatine/creatinine ratio in urine was elevated only in three of eight females. (ii) Although as a group the females had a significantly decreased cerebral creatine concentration, individual females had creatine concentrations overlapping with normal controls. (iii) Skewed X-inactivation was found in the cultured fibroblasts, in favour 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 defect. We conclude that testing for creatine transporter defect should be considered in females with (mild) mental retardation. Screening by DNA analysis of the *SLC6A8* gene is recommended.

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Key words: cerebral creatine deficiency – female carrier – SLC6A8 – spectroscopy – X-linked mental retardation

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Received 13 January 2010, revised and accepted for publication 3 May 2010

The creatine transporter defect is an X-linked cause of mental retardation with a prevalence of 0.3–3.5% in males (1–5). The first male patient with creatine transporter defect was described in 2001 (6, 7). Since then several male patients have been reported (8–20). Patients present with mental retardation, severe speech delay, behaviour

disturbances and epilepsy. The X-linked creatine transporter defect 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 ^1H -magnetic resonance spectroscopy (MRS) of the brain. An increased creatine/creatinine ratio in urine is used as a marker for the diagnosis of the creatine transporter defect in male patients (21, 22), although this test has a high rate of false positive results (3, 23). DNA analysis of the *SLC6A8* gene and creatine uptake studies in cultured fibroblasts are used to confirm the diagnosis (24).

Because the creatine transporter defect is an X-linked condition, the phenotype in females is expected to be influenced by the X-inactivation pattern. Learning difficulties or mild mental retardation has been mentioned in several heterozygous females in the reported creatine transporter defect families but with few clinical details (7, 8, 11–18, 20, 25, 26). We report the systematic study of clinical features and X-inactivation pattern in heterozygous females in eight Dutch creatine transporter defect families to answer the following questions: (i) Do females who are heterozygous for the creatine transporter defect present with symptoms? (ii) How do we diagnose heterozygous females? (iii) What is the X-inactivation pattern in heterozygous females and is there a correlation with the phenotype?

Methods

Subjects

Twelve index boys with a creatine transporter defect were diagnosed in the Netherlands till 2007. Nine of 11 mothers tested for the mutation were found to be (non-mosaic) carriers and one mother had a low-level somatic mosaicism (27). In addition, two sisters of index boys were found to be heterozygous. All heterozygous females were diagnosed by DNA analysis.

All 11 non-mosaic heterozygous females were invited to participate in the study of which three declined. Eight heterozygous females, aged 32–77 years (mean age 47 years), from eight families were included in this study. All participants gave informed consent. This 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 neurological 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 (28). Education was categorized according to the system of Verhage (29).

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. [21]. Creatinine (Crn) in urine was measured by the Jaffé method.

Magnetic resonance imaging and spectroscopy

Magnetic resonance examinations were performed at 1.5 T (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 three-dimensional T1-weighted sagittal images. In each VOI, a fully relaxed, short-echo time stimulated echo acquisition mode (STEAM) spectrum (repetition time/echo time/mixing time = 6000/20/10 ms; 64 acquisitions) was obtained and spectra were quantified using LCModel (30). In this study, concentrations of total NAA (the sum of *N*-acetylaspartate and *N*-acetylaspartyl glutamate), total Cr (the sum of creatine and phosphocreatine), Cho (choline-containing compounds), and Ins (myo-Inositol) were considered, and expressed in millimolar per litre 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.

Creatine uptake assay

The creatine uptake assay in cultured skin fibroblasts was performed according to Rosenberg et al. (24). Creatine uptake was measured after incubation with 25 μM 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

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polymorphic (CAG)_n repeat in the first exon of the androgen receptor (*AR*) gene with and without digestion of the DNA with the methylation-sensitive enzyme *HhaI* (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, Nieuwerkerk aan de IJssel, The Netherlands) 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: % inactive A1 = $100 \times (A1^+/A1^-)/[(A1^+/A1^-) + (A2^+/A2^-)]$, where A1 and A2 represent the smaller and the larger alleles and + and - represent the digested and undigested samples, respectively (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 *SLC6A8* gene was performed according to Rosenberg et al. (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. Mental retardation was evident in one female (individual 1). One other female required special education and three females failed a year at elementary school. Most females had an educational level score according to Verhage (29) of 4 or 5, which is average.

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

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 (26).

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 (16).

Physical examination

No consistent evident dysmorphisms were detected. Body mass index 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 SD 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 mental retardation range (IQ < 70) and four females in the range of borderline intellectual functioning (IQ 70–85).

Biochemical analysis

GAA in urine and plasma was in the normal range (21) in all females. The creatine/creatinine ratio in urine was mildly elevated in three females (Table 1). Two females also had a mildly elevated creatine in plasma.

Magnetic resonance imaging and spectroscopy

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 (Fig. 1). Individual results are summarized in Table 1 as percentage of normal (measured value/mean of controls $\times 100$) averaged over the three regions. For the other metabolites,

Table 1. Clinical features of heterozygous females

Individual	Age (years)	Development	Learning difficulties	Other symptoms	Neurological examination	Verhage ^a	IQ 95% CI	Urine Cr/Crn	Plasma Cr (μmol/l)	tCr brain (% normal mean)	Mutation
1	42	Mild MD/SD, PT, ST	Special education sheltered workplace	Possibly seizures at 12–14 years	Mild cerebellar symptoms	3	41–55	0.679	91	66	c.1495+5G>C
2	65	Normal	Failed a year in primary school	Severe constipation	Normal	4	66–80	0.337	44	70	c.1011C>G
3	40	Normal	No	Breast cancer at 38 years	Normal	5	65–80	0.098	44	87	p.(Cys337Trp) c.1631C>T
4	41	Normal, ST	Special education	EEG for unknown reason at 10 years Breast cancer at 41 years	Normal	4	65–79	0.384	62	78	p.(Pro544Leu) c.570_571del
5	43	Normal	Failed a year in primary school		Normal	4	65–79	0.059	38	87	p.(Ala191GlnfsX10) c.428_430del
6	32	Walking at 2 years	No	Irritable bowel syndrome with constipation	Mild cerebellar symptoms	5	78–92	0.065	30	83	p.(Tyr143del) c.92delC
7	35	Normal	No		Normal	5	89–103	0.057	35	82	p.(Pro31ArgfsX66) c.778-300_1764del
8	77	Normal	Failed a year in primary school		Normal	3 ^b	53–67	0.051	31	65	c.1299_1309del
Normal controls								0.011–0.244 ^c	6–50 ^c		p.(Pro434LeufsX27)
Affected boys								1.4–5.5 ^c			

CI, confidence interval; Cr, creatinine; Crn, creatinine; EEG, electroencephalogram; IQ, intelligence quotient; MD, motor delay; PT, physical therapy; SD, speech delay; ST, speech therapy.
^aVerhage scoring system [29]: 1, not 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.

^bSecondary school not finished because of World War II.

^cFrom Almeida et al. (21).

Table 2. Metabolite concentrations obtained from ¹H-magnetic resonance spectroscopy (mean ± SD, in mM) in cortex, white matter and cerebellum of heterozygous females vs controls

	Cortex			White matter			Cerebellum		
	Heterozygotes (n = 8)	Control (n = 24)	p	Heterozygotes (n = 8)	Control (n = 15)	p	Heterozygotes (n = 8)	Control (n = 13)	p
tCr	4.5 ± 0.8	5.8 ± 0.4	<0.01	3.7 ± 0.3	4.65 ± 0.50	<0.001	5.6 ± 0.7	7.4 ± 0.8	<0.001
tNAA	8.5 ± 0.7	7.8 ± 0.5	<0.05	7.9 ± 1.2	8.00 ± 0.64	ns	7.4 ± 0.8	7.0 ± 0.6	ns
Cho	0.89 ± 0.10	0.96 ± 0.11	ns	1.43 ± 0.18	1.38 ± 0.12	ns	1.56 ± 0.26	1.73 ± 0.15	ns
Ins	3.8 ± 0.5	3.8 ± 0.4	ns	3.7 ± 0.5	3.6 ± 0.6	ns	4.3 ± 0.7	4.6 ± 0.6	ns

Cho, choline-containing compounds; Ins, myo-inositol; ns, not significant; tCr, total creatine; tNAA, total *N*-acetylaspartate.

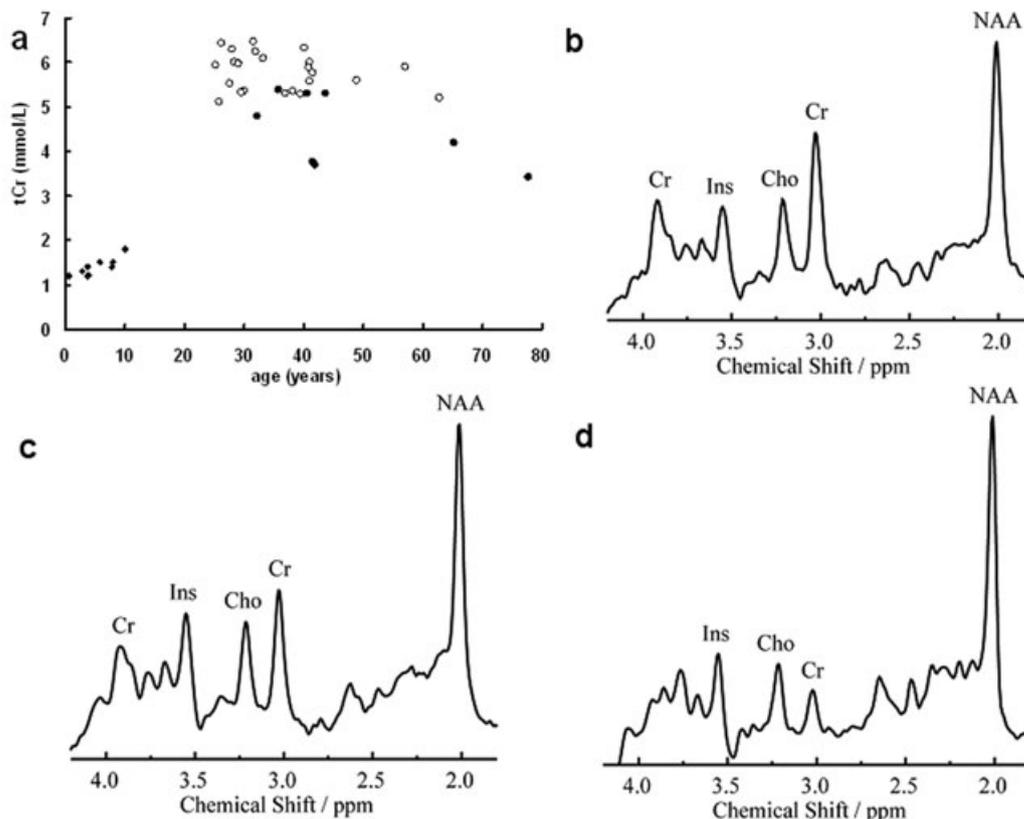


Fig. 1. (a) Total creatine concentration in the cortex of heterozygous females (black dots) plotted as a function of age, compared to normal controls (open dots) and affected male patients (black diamonds). ¹H-magnetic resonance spectroscopy STEAM spectrum in cortex of a normal control (b), a heterozygous female (c) and an affected male patient (d). NAA, *N*-acetylaspartate; Cr, creatine and phosphocreatine; Cho, choline-containing compound; Ins myo-inositol.

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.

Creatine uptake assay

Creatine uptake assay in cultured skin fibroblasts was performed in seven of eight heterozygotes because the culture of skin fibroblasts failed in one female. Results were compared with uptake assays

in 12 male SLC6A8-deficient patient fibroblasts and 13 normal control fibroblasts (24) (Fig. 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).

X-inactivation studies

One heterozygous female (individual 8) was uninformative for the (CAG)_n repeat polymorphism

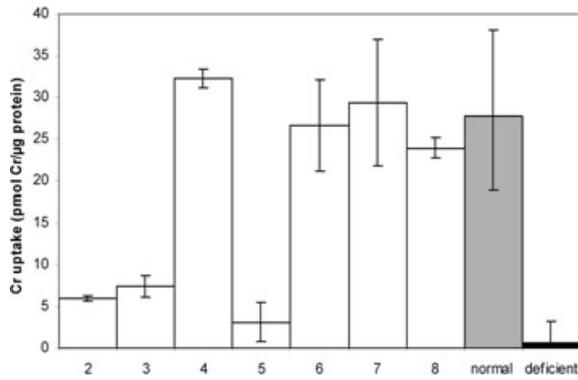


Fig. 2. Creatine uptake in skin fibroblasts of heterozygous females (white, numbered), normal controls (grey) and deficient controls (black) with incubations of 25 µM creatine. The values represent the mean ± SD of triplicate incubations of the heterozygous females and the mean + range of 13 normal controls and 12 deficient controls.

and the X-inactivation pattern could therefore not be determined. X-inactivation patterns of the other seven females are shown in Fig. 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 and the mutated alleles 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 favour of the wild-type allele and she had the highest IQ score in this study. In the other (individual 5), the skewing was in favour of the mutated allele but she did not have a more pronounced abnormal phenotype.

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 Fig. 4. A Pearson correlation of respectively 0.65 ($r^2 = 0.43$) and -0.64 ($r^2 = 0.40$) was found. These correlations are however not significant.

Discussion

We detected symptoms of the creatine transporter defect in female heterozygotes. Mild mental retardation was evident in one heterozygote with developmental delay, learning difficulties requiring

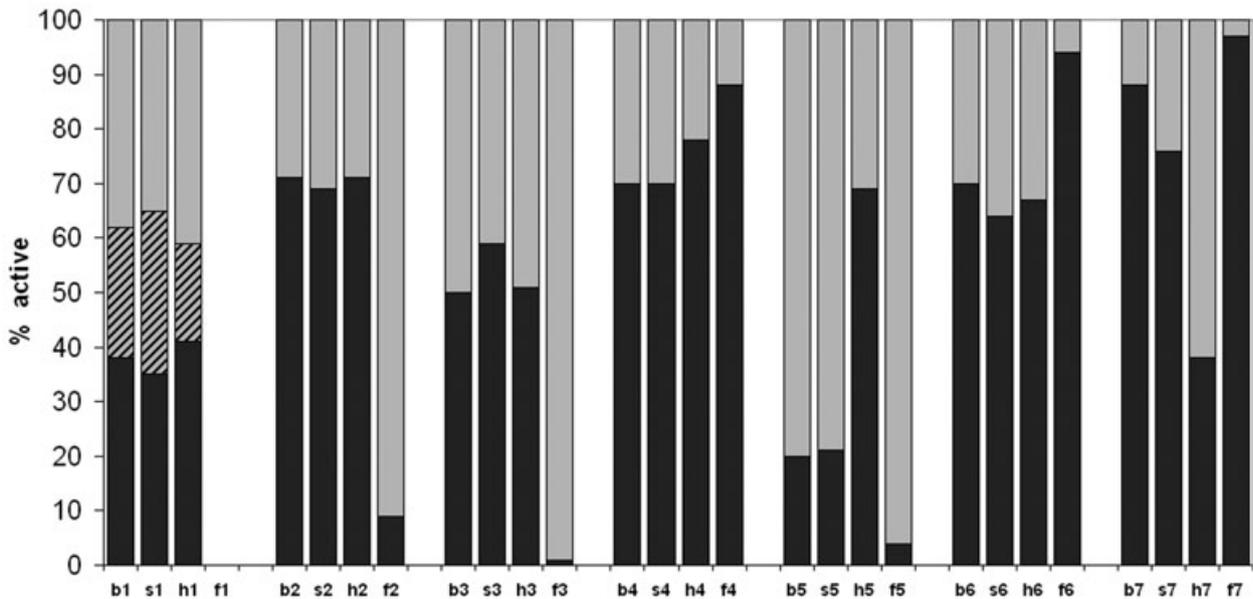


Fig. 3. X-inactivation in blood leukocytes (b), saliva (s), hair roots (h) and cultured skin fibroblasts (f) in seven heterozygous females (1–7). The percentage of active wild-type allele is shown in black and of active mutated allele in grey. In individual 1, it is unknown which is the wild-type allele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

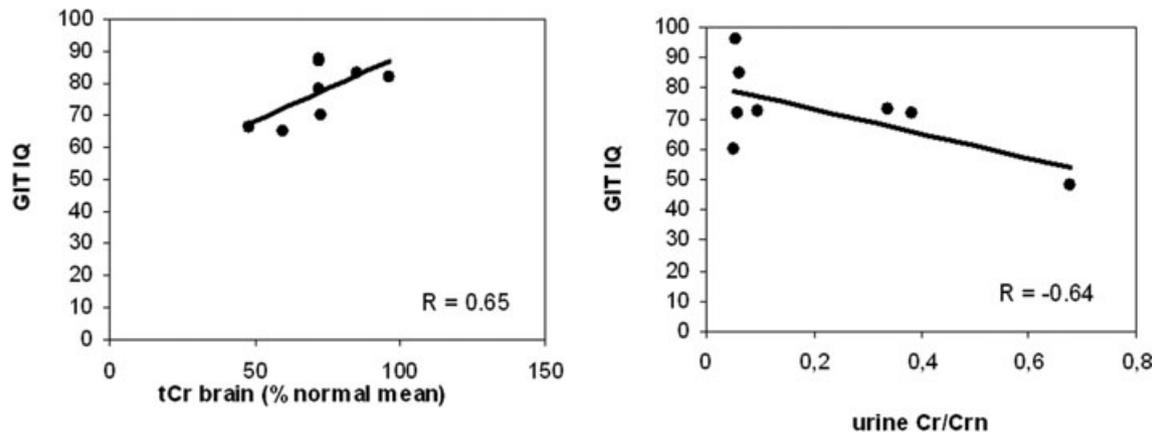


Fig. 4. Correlations between IQ scores and respectively total creatine concentrations in the brain and urinary creatine/creatinine ratio.

special education and an IQ score well below 70. IQ scores were <70 (mental retardation 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) (33), 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. The severe constipation from the age of 55 in a 65-year-old female is remarkable. Gastrointestinal problems including chronic constipation, megacolon, ulcer disease, ileus and bowel perforation have been described in adult males with the creatine transporter defect, which include two brothers of the heterozygous female with severe constipation in this study (15, 16). Severe constipation and ileus might be a complication of the creatine transporter defect that develops later in life and can affect heterozygous females as well. Breast cancer was diagnosed at a relatively younger age in two heterozygous females. We could not relate this with the creatine transporter defect.

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

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 only in three of eight females in this study and the elevation was very mild. Creatine depletion on ^1H -MRS of the brain is a hallmark of the disease in male creatine transporter defect 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 ^1H -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 of seven females and could thus confirm the diagnosis but the other four had a normal uptake.

DNA analysis of the *SLC6A8* gene (open reading frame 1.9 kb, 13 exons) is most likely the only reliable option for screening for a creatine transporter defect in females presenting with (mild) mental retardation. DNA testing for creatine transporter defect is currently not systematically included in the diagnostic workup of females with mental retardation.

Some X-linked mental retardation 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 (34, 35). We did not find consistent skewing in peripheral blood leukocytes, hairs and saliva, indicating that there is no selection against cells with a creatine transporter defect. 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 favourably to unfavourably skewed (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 in favour of both the mutated and the wild-type alleles. We hypothesize that the selection is directed at another, unrelated, factor. Likewise, Plagnol et al. showed that culturing in lymphoblastoid cell lines often leads to mono- or pauciclinality (37). Importantly, this severe skewing in cultured fibroblasts makes the study of creatine uptake in cultured fibroblasts unreliable for the diagnosis of the creatine transporter defect in female heterozygotes.

In conclusion, this study shows that females who are heterozygous for the creatine transporter defect can have symptoms of this condition. Testing for this condition should be considered in females with (mild) mental retardation but screening based on urinary creatine/creatinine ratio is not reliable and

screening with DNA analysis of the *SLC6A8* gene is recommended.

Acknowledgements

We thank the families for their participation in this study. We thank T Kleefstra, A Maat-Kievit and SG Frints. for their help with the inclusion of families and FK Aarsen for her useful suggestions on the neuropsychological assessments. The work of G. S. S. was supported by the Dutch society for Scientific Research (ZonMW/NWO), VIDI Grant No. 917.56.349.

Conflict of interest

The authors have no conflict of interest.

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