

## ORIGINAL ARTICLE

## Phenotype and genotype in 101 males with X-linked creatine transporter deficiency

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**ABSTRACT**

**Background** Creatine transporter deficiency is a monogenic cause of X-linked intellectual disability. Since its first description in 2001 several case reports have been published but an overview of phenotype, genotype and phenotype–genotype correlation has been lacking.

**Methods** We performed a retrospective study of clinical, biochemical and molecular genetic data of 101 males with X-linked creatine transporter deficiency from 85 families with a pathogenic mutation in the creatine transporter gene (*SLC6A8*).

**Results and conclusions** Most patients developed moderate to severe intellectual disability; mild intellectual disability was rare in adult patients. Speech language development was especially delayed but almost a third of the patients were able to speak in sentences. Besides behavioural problems and seizures, mild to moderate motor dysfunction, including extrapyramidal movement abnormalities, and gastrointestinal problems were frequent clinical features. Urinary creatine to creatinine ratio proved to be a reliable screening method besides MR spectroscopy, molecular genetic testing and creatine uptake studies, allowing definition of diagnostic guidelines. A third of patients had a de novo mutation in the *SLC6A8* gene. Mothers with an affected son with a de novo mutation should be counselled about a recurrence risk in further pregnancies due to the possibility of low level somatic or germline mosaicism. Missense mutations with residual activity might be associated with a milder phenotype and large deletions extending beyond the 3' end of the *SLC6A8* gene with a more severe phenotype. Evaluation of the biochemical phenotype revealed unexpected high creatine levels in cerebrospinal fluid suggesting that the brain is able to synthesise creatine and that the cerebral creatine deficiency is caused by a defect in the reuptake of creatine within the neurones.

**INTRODUCTION**

In 2001, a 6-year-old boy with intellectual disability (ID), severe speech delay, mild hypotonia, short attention span and status epilepticus was evaluated. The severely reduced cerebral creatine signal on proton MR spectroscopy (<sup>1</sup>H-MRS) in combination with a family history suspect for X-linked inheritance and an increased creatine to creatinine (Cr to Crn) ratio in urine led to the discovery of the X-linked creatine transporter deficiency (CRTR-D).<sup>1–2</sup> Creatine uptake in cultured skin fibroblasts was deficient and a hemizygous nonsense mutation was detected in the *SLC6A8* gene, which is located on the X-chromosome. CRTR-D comprises together with the autosomal recessive creatine biosynthesis defects arginine:glycine amidinotransferase (AGAT) deficiency and guanidinoacetate methyltransferase (GAMT) deficiency, the cerebral creatine deficiency syndromes.

Shortly after the description of the first patient, more patients were diagnosed and the prevalence of the disorder was estimated between 0.3% and 3.5% in males with ID<sup>3–7</sup> and around 2% in X-linked ID.<sup>3–7</sup> Several case reports have been published;<sup>6–8 8–36</sup> however, an overview has been lacking.

Creatine is mainly known for its essential role in energy metabolism as the creatine/phosphocreatine system serves as a buffer in the regeneration of ATP and as a shuttle of high-energy phosphates between mitochondrial sites of production to cytosolic sites of utilisation.<sup>37</sup> However, creatine might also have a neuromodulatory role.<sup>38</sup> Creatine is derived from the diet and endogenous synthesis from arginine and glycine. Originally, it was thought that the first step of creatine synthesis (catalysed by AGAT), yielding guanidinoacetate (GAA), took place mainly in the kidney and the second step (catalysed by GAMT) in the liver and that creatine was taken from the blood by creatine-requiring tissues.

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## Developmental defects

However, the expression of AGAT and GAMT enzymes seems to be more widespread and the contribution of various tissues to creatine synthesis is still unclear.<sup>37</sup>

Here we present an overview of the clinical spectrum of the CRTR-D with clinical, biochemical and molecular genetic data of a large cohort including 101 males from 85 families. This overview provides directions for the management of patients and counselling of families regarding prognosis and recurrence risks. Diagnostic guidelines are proposed and possible genotype–phenotype relations are discussed. The study also reveals important clues about the disease patho-mechanism and creatine metabolism.

### METHODS

#### Collection of clinical and biochemical data

The VU University Medical Centre (VUMC), Amsterdam, was the first centre performing molecular genetic analysis of the *SLC6A8* gene. Until October 2012, a pathogenic mutation had been detected in 109 male probands from all over the world. Comprehensive questionnaires were sent to the physicians of male patients with a pathogenic mutation in the *SLC6A8* gene. Additionally, all male patients published up to October 2012 as case reports were included. Female heterozygotes were not included in this study.

The study was approved by the ethics committee of the VUMC, Amsterdam, The Netherlands.

#### Analysis of the *SLC6A8* gene

All 13 exons of the *SLC6A8* gene (NM\_005629.3) were sequenced as previously described<sup>39</sup> at the VUMC in 87 of the 103 patients in this cohort. DNA analysis in the other 16 patients was performed in various other laboratories.

In four cases a large deletion was detected. This was confirmed by multiplex ligation-dependent probe amplification using the P049 kit according to standard protocol (MRC-Holland, The Netherlands). The breakpoints were amplified by long range PCR and confirmed by direct DNA sequence analysis.

Novel missense variants were further characterised by testing for restoration of creatine uptake in CRTR deficient fibroblasts after transfection with *SLC6A8* cDNA in which the variant had been introduced by site-directed mutagenesis.<sup>40 41</sup> Novel neutral or intronic variants were further characterised by bioinformatics analysis and mRNA analysis.<sup>39</sup>

#### Creatine uptake in skin fibroblasts

The creatine uptake after incubation at 25  $\mu$ M creatine was measured as previously described.<sup>41</sup>

#### Statistical analysis genotype–phenotype correlation

An unpaired t test was applied for statistical analysis.

### RESULTS

#### Patients

Questionnaires of 77 male patients from 64 families were received. A total of 30 of these 77 patients were also reported in previous case reports or treatment trials.<sup>7 10 12 13 15 16 20–23 26 27 29 31 42</sup> Two patients were excluded because of the presence of a large deletion including neighbouring gene(s).<sup>12 31</sup> Additionally 26 previously reported male patients from 23 families<sup>1 2 6–9 11 12 14 17–19 22 24 26 28 30 32–36</sup> were included. In total 101 male patients from 85 families were included. Age varied between 1 and 66 years, with median age of 10 years (n=97). Twenty-one patients were adults (> 18 years).

### Phenotype

#### Presentation

The patients came to medical attention between birth and 6 years (n=82; mean 1 year). The presenting symptoms are shown in table 1.

#### Development delay

Patients achieved independent walking at a mean age of 2 years (n=67; range 13 months–4 years). Speech development was delayed in all. First words were at a mean age of 3.1 years (n=44; range 9 months–10 years). The level of speech according to age is shown in figure 1.

ID was classified as severe (IQ 20–34), moderate (IQ 35–49) or mild (IQ 50–69) based on neuropsychological testing if performed (n=48) or as estimated by the referring physician (n=43). The ID was moderate to severe in adult patients (figure 2); only one 22-year-old male had mild ID (IQ 69).<sup>20</sup> One adult had progressive cognitive dysfunction.<sup>15</sup>

#### Behaviour problems

These were mentioned in 85% of patients. Attention deficit and/or hyperactivity (55%) and autistic features (41%) were the most common behaviour problems followed by social anxiety/shyness (20%), stereotypic behaviour (20%), impulsive behaviour (27%), aggressive behaviour (19%), self-injurious behaviour (10%) and obsessive compulsive behaviour (8%).

#### Seizures

Seizures were present in 59% of patients. Seizures were infrequent and well controlled with antiepileptic medication in most patients. The most common seizure types were generalised tonic-clonic seizure and simple or complex partial seizures with or without secondary generalisation. Absence or myoclonic seizures occurred in a few patients. Patients often had febrile seizures in addition to non-febrile seizures. Three patients had severe refractory epilepsy. Status epilepticus occurred in nine patients. Mean age of onset for non-febrile seizures was 4.5 years (n=41; range 1–21 years).

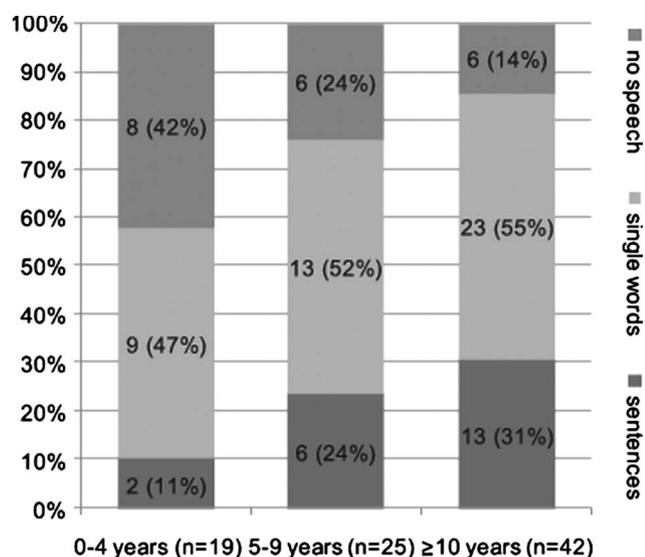
#### Other neurological symptoms

Motor dysfunction, mostly mild to moderate, was mentioned in 58%. Hypotonia was most common (40%) and often improved with age. Other findings were signs of spasticity (stiff gait, mildly increased tonus, mildly increased reflexes) in 26%, coordination dysfunction (wide based/unstable gait, dysarthria, ataxia, clumsiness) in 29% and dystonia or athetosis (abnormal

**Table 1** Symptoms at first presentation (n=87)

Sign	Present in %
Developmental delay	80
Hypotonia	10
Gastrointestinal	16
Failure to thrive/growth delay/poor weight gain	8
Vomiting	5
Gastrointestinal reflux	2
Feeding difficulties/poor suck and swallowing	5
Seizures	10
Behavioural problems	6
Other (clubfoot, strabismus, adducted thumbs and laryngomalacia, PVCs, apnoea)	6

PVCs, premature ventricular contractions.



**Figure 1** Level of speech according to age groups. In the columns, the number of patients and the per cent of total number of patients are shown.

hand movements, intermittent dystonic posturing of the hands/wrists during walking, choreathetoid movements, dystonia of face and upper limbs) in 11%.

#### Additional symptoms

Gastrointestinal symptoms were reported in 35% and consisted of neonatal feeding difficulties (13%), failure to thrive (16%), vomiting (11%), (severe) chronic constipation (13%, nine out of 13 were adults), ileus (3%), hepatitis (n=1), gastric and duodenal ulcers (n=2) and hiatal hernia (n=1).

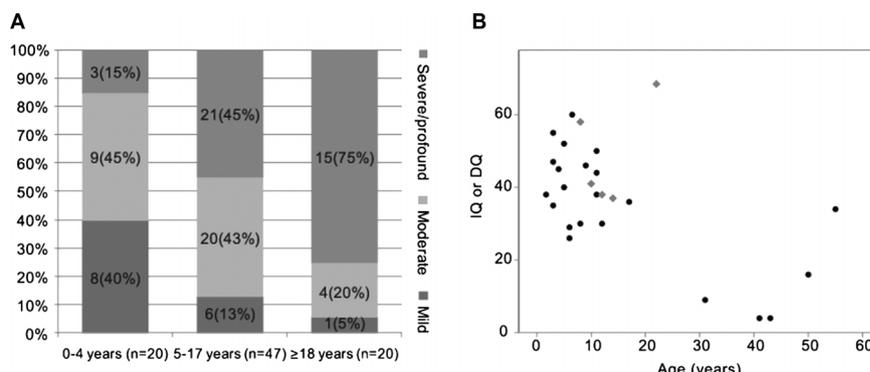
Urogenital anomalies were reported in 9% and consisted of dysfunctional voiding (3%), bladder instability, recurrent urinary infections, urethra stenosis, bilateral renal cysts and unilateral testis hypoplasia (all n=1).

Ten patients (10%) had ophthalmological abnormalities including strabismus (7%), bilateral abducens nerve palsy or Duane anomaly (n=2), mild cerebral visual deficit and probably traumatic unilateral cataract and blindness (both n=1).

Four patients had mild (sensorial-neural) hearing loss.

Two previously described patients had mild cardiomyopathy.<sup>20</sup> One patient had multiple premature ventricular contractions.<sup>33</sup> One patient had long QT syndrome (QT time 495 ms, reference 350–440).

**Figure 2** Degree of intellectual disability according to age groups (A) and Developmental Quotient (DQ) or Intelligence Quotient (IQ) scores for age (B). (A) In the columns, the number of patients and the per cent of total number of patients are shown. (B) Exact DQ or IQ scores with ages were known in 27 patients (if more than one evaluation was known, the last was used). The grey diamonds depict patients with a missense mutation with residual activity. It should be noted that the four lowest IQs (scores 4–16) in the adults were brothers.<sup>10</sup>



#### Survival

Three patients, who were brothers, died: one at 21 years from tuberculosis and two around 40 and 60 years of unknown cause.

#### Family history

The family history was positive for X-linked ID (defined as ID in male relatives in the maternal line) in 20/85 (24%) index patients of which 18 had one or more (half-) brother(s) with ID. Five index patients had one or more (half-) sister(s) with ID. The mothers of 18/85 (21%) index patients had learning difficulties/mild ID of which 14 were proven heterozygotes and two had two or more affected sons but were not confirmed heterozygotes since no DNA analysis was performed.

#### Physical examination

Most patients had below average height while head circumference varied between +2 and -2 SD (see online supplementary figure). Variable dysmorphic features were mentioned in 45% of the patients including broad/prominent forehead, mid-face hypoplasia, myopathic facies, ptosis, short nose, simple/unfolded/large ears and joint laxity. Slender build and/or poorly developed muscular mass were frequent but not consistent signs.

#### Neuroimaging and MRS

Brain MRI was available in 76 patients and showed (mild) abnormalities in 53 patients, including mildly delayed myelination, (T2-) hyperintensities, thin corpus callosum, mildly enlarged ventricles/extracerebral spaces and cerebral/cerebellar atrophy. Cerebral atrophy was progressive in two brothers.<sup>8</sup>

MRS results were available in 66 patients showing total absence or severe reduction of creatine.

#### Biochemical analysis

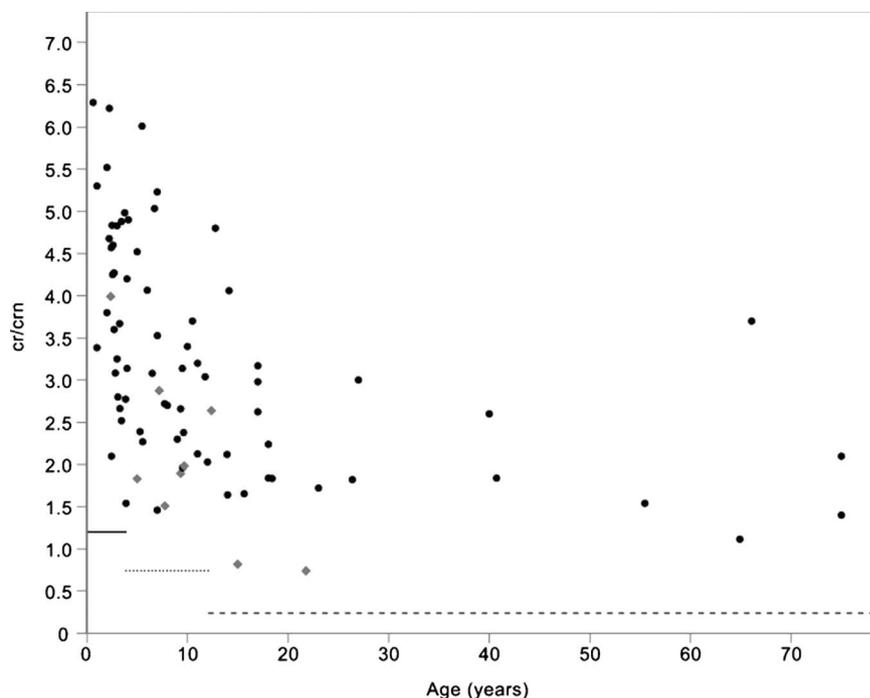
The quantitative urinary Cr to Crn ratio was available in 81 patients and was elevated in all (figure 3). Additionally, six patients were said to have elevated levels.

Creatine in plasma was available in 28 patients: 21 patients <10 years with normal levels between 67 and 105  $\mu\text{M}$ , mean 90  $\mu\text{M}$  (reference 17–109  $\mu\text{M}$ <sup>43</sup>) and seven patients >10 years with elevated levels between 60 and 103  $\mu\text{M}$ , mean 82  $\mu\text{M}$  (reference 6–50  $\mu\text{M}$ <sup>43</sup>).

Creatine in cerebrospinal fluid (CSF), available in seven untreated patients, varied between 51 and 80  $\mu\text{M}$  (mean 67  $\mu\text{M}$ ) and was elevated in four (reference range 24–66  $\mu\text{M}$ <sup>43</sup>). Additionally, one patient had normal CSF creatine (62  $\mu\text{M}$ ) during creatine supplementation.

## Developmental defects

**Figure 3** Urinary creatine to creatinine ratio shown for age in 81 patients (if more than one measurement was known, the lowest value was depicted). The grey diamonds depict patients with a missense mutation with residual activity. Upper limit of normal range for age <4 years (1.2) is depicted with uninterrupted line, for age 4–12 years (0.74) with small dashes and for age >12 years (0.24) with large dashes.



GAA in CSF, available in six untreated patients, was normal or slightly elevated (0.05–0.44  $\mu\text{M}$ , mean 0.18  $\mu\text{M}$ ; reference range 0.036–0.22  $\mu\text{M}$ <sup>43</sup>).

Creatinine in CSF was available in three (12–15  $\mu\text{M}$ , mean 13  $\mu\text{M}$ ) and decreased in all (reference range 29–41  $\mu\text{M}$ <sup>11</sup>). Mildly or transient plasma lactate elevations and increased urinary 3-methylglutaconic acid and/or ethylmalonic acid and tricarboxylic acid cycle intermediates were mentioned in two patients with normal respiratory chain complex activities in a muscle biopsy.<sup>12 27</sup>

#### Creatine uptake in fibroblasts

We performed creatine uptake studies in cultured skin fibroblast in 41 patients and all had deficient uptake. Uptake expressed as per cent of uptake in control cell line used in the same test varied between 0% and 10.5% (mean 3.6%). Additionally, six patients were reported to have deficient uptake studies.

#### Diagnosis

Patients were detected by urinary, MRS or DNA investigations (table 2). The diagnosis was confirmed by DNA analysis of the *SLC6A8* gene in all patients.

#### Treatment

Twenty-one patients were included in previously published trials with L-arginine, alone or in combination with creatine and/or glycine. No substantial clinical effect or increase in cerebral creatine was seen in most patients<sup>21 42 44</sup> although improvements were reported in single cases.<sup>34 35</sup> In addition, 26 patients were treated with either creatine monotherapy (n=5), a protocol including L-arginine (n=12) or an unspecified protocol (n=8). Data are lacking to assess the effectiveness in these patients.

#### Genotype

The most common mutation types were missense mutations (23 different mutations in 26/85 (31%) families) and one-amino acid (3 bp) deletions (nine mutations in 20/85 (24%) families). Most missense and 3 bp deletions occurred in TM7 and TM8

(figure 4). The pathogenicity of 20 out of the 23 missense mutations was confirmed by overexpression studies in CRTR deficient fibroblasts (see online supplementary table). Notably, four missense mutations, c.1271G>A; p.(Gly424Asp), c.1661C>T; p.(Pro544Leu), c.1699T>C; p.(Ser567Pro) and c.1190C>T; p.(Pro397Leu) had residual CRTR activity.

Less common mutation types were frameshift mutations (13 mutations in 15 families), nonsense mutations (nine mutations in 11 families), splice error mutations (intronic or synonymous variants with aberrant splicing by mRNA analysis and/or splice-site analysis tools) (seven mutations in nine families) and a translation initiation site mutation, c.1A>G; p.(Met1?) (one family).

In four unrelated patients, large multiple-exon deletions were detected, including two previously reported patients<sup>12</sup> of whom the breakpoints were now clarified. Two deletions extended in neighbouring gene(s) and these patients were excluded from this study. The other two patients had deletions of exons 5–12 and exons 8–13, respectively.

In total, 65 different mutations (see online supplementary table) were identified. Ten mutations reoccurred in two or more families. The two most common mutations were c.1222\_1224del; p.(Phe408del) detected in seven and c.1006\_1008del; p.(Asn336del) in five unrelated families. All mutations are included in the Leiden Open Variation Database (LOVD) (<http://www.LOVD.nl/SLC6A8>).

Molecular genetic studies were performed in 61 mothers and the mutation was de novo in 18 (30%) sons. A low level mosaicism was found in four mothers (7%). Three grandmothers were heterozygous for the family mutation.

#### Genotype–phenotype correlation

Nine patients had a missense mutation with residual CRTR activity. Four had mild ID and five moderate ID. IQ scores were relatively high (n=6, 55±19) compared with the rest of the cohort (35±15) although this was not significant (p=0.055). Six patients (aged 7–22 years) spoke in sentences and three (aged 3–16 years) spoke single words. All patients had

**Table 2** Screening methods: initial diagnostic tests leading to the diagnosis in 101 patients

Initial test	%
Urinary Cr to Crn ratio	39
MRS	21
Urinary Cr to Crn ratio and MRS	4
DNA testing familial mutation	14
DNA sequencing*	10
Unknown	12

\*Performed in research cohorts of (X-linked) intellectual disability (n=9) or because of suspicion of CRTR-D based on clinical features (n=1).

Cr to Crn, creatine to creatinine; CRTR-D, creatine transporter deficiency; MRS, MR spectroscopy.

behavioural problems but they were mild in three. Two patients had seizures, one with severe refractory epilepsy.<sup>17</sup>

The urinary Cr to Crn ratio in these nine patients was significantly lower compared with the remainder of the cohort ( $2.0 \pm 1.0$  vs  $3.3 \pm 1.3$ ;  $p=0.005$ ) while age at urinary sampling between these two groups was not significantly different. The creatine uptake in fibroblasts was significantly higher in four of the five patients of whom fibroblasts were available compared with the rest of the cohort ( $9.3\% \pm 1.4\%$  vs  $3.1\% \pm 2.9\%$ ;  $p=0.0001$ ) but one patient (c.1190C>T; p.(Pro397Leu)) had almost no uptake (1%).

The patient with exons 5–13 deletion had a severe phenotype with severe failure to thrive, severe motor delay, profound hypotonia, dystonia and choreo-athetoid movements.<sup>12</sup> The patient with exons 5–12 deletion had a moderate phenotype without movement disorder. Urinary Cr to Crn ratio was 3.8 and 1.8, respectively.

Urinary Cr to Crn ratio and creatine uptake in fibroblast did not differ significantly between patients with truncating or non-truncating mutations.

## DISCUSSION

### Phenotype

This survey of 101 male patients with XL-CRTR-D confirms that the most common clinical features are ID with prominent speech delay, behavioural abnormalities and seizures, but also allows new and more detailed conclusions regarding the phenotype. Female heterozygotes were not included in this study since the phenotype in females is largely dependent on the individual X-inactivation pattern causing a wide variation in phenotype.<sup>25 45</sup>

The degree of ID varied from mild to severe with IQ scores usually between 25 and 60. It should be noted that the IQ scores are based on a limited number of patients and are probably biased towards the higher functioning patients as

assessment of severely affected patients is often not initiated. Mild ID was mainly restricted to younger patients with the exception of one adult with a missense mutation with residual transporter activity.<sup>20</sup> Most adults had severe ID suggesting that patients generally develop moderate to severe ID. However, mildly affected patients might remain more often undiagnosed.

The more pronounced ID in adults is probably due to the increasing gap between chronological and developmental age which causes a decline in IQ scores. We previously noted a similar age-related decline in IQ subscores, without regression, in the follow-up of children with CRTR-D.<sup>44</sup> Profound ID with IQ scores <16 were found in four middle aged brothers (31–50 years)<sup>10</sup> and a progressive course of ID has been suggested.<sup>10 15</sup> However, evident cognitive regression was found only in one<sup>15</sup> of the 21 adult patients in our cohort. Repeated neuropsychological assessments should be performed in adult patients to determine whether cognitive dysfunction is a progressive process.

Patients were especially delayed in speech development while motor development was only mildly delayed. Although most patients had speech limited to single words, almost a third of the patients >10 years spoke in sentences.

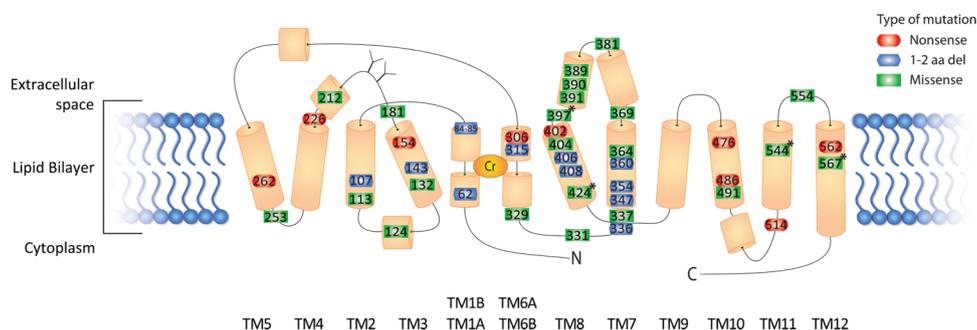
Other common symptoms were behavioural problems (85%), mainly autistic features and attention deficit and hyperactivity, and seizures (59%) which were often infrequent and easily controlled and included febrile seizures. However, status epilepticus occurred and some patients had severe refractory epilepsy.

Hypotonia, mild signs of spasticity and coordination disturbances were quite common in our cohort. Extrapyramidal movement abnormalities, for example, dystonia and athetosis, are commonly associated with GAMT deficiency<sup>46</sup> but occurred in 11% of the CRTR-D patients in our cohort, although mostly mild. Unspecified myopathic symptoms were noted in only a few patients.

Gastrointestinal problems occurred frequently in CRTR-D patients. Feeding difficulties, vomiting and failure to thrive were early and sometimes first presenting symptoms. Severe constipation and ileus developed later in life as previously described.<sup>10 15</sup> This might be a consequence of smooth muscle problems or autonomic nerve dysfunction which might also affect bladder function. Bladder voiding dysfunction or instability was noticed in a few patients. Caregivers should be alert to these problems, especially in older CRTR-D patients.

The below average height and slender build of most patients might be due to gastrointestinal dysfunction but could also be a consequence of cerebral or intracellular creatine depletion. Weight gain is the only consistent side effect of creatine supplementation and most likely reflects an increase in muscular mass either due to increased muscle protein synthesis or water retention in the initial days.<sup>37</sup> Cerebral creatine might also have an effect on appetite<sup>47</sup> and growth hormone secretion.<sup>48</sup>

**Figure 4** Locations of missense and 3 bp deletions in *SLC6A8* protein. Missense mutations with a residual activity are marked by an asterisk (\*).



## Developmental defects

Only four patients had (mild) cardiac abnormalities and retinal abnormalities were not found in this cohort. It is remarkable that the tissues with under physiological circumstances the highest creatine content, including skeletal muscle, heart and retina,<sup>37</sup> seem to be mostly unaffected in CRTR-D. Skeletal muscle and heart have, together with kidneys, the highest CRTR expression<sup>37 49</sup> and CRTR is also expressed in the retina.<sup>50 51</sup> There are several possible explanations. First, abnormalities might be underdiagnosed because most patients did not undergo ophthalmological and cardiac investigations. The symptoms might also present later in life. Involvement of these organs should be evaluated in a large patient cohort and physicians should be aware of the possible involvement in CRTR-D patients. Second, it is possible that these tissues acquire creatine in a different way, possibly by endogenous creatine synthesis. This implies that creatine synthesis in the creatine-requiring tissues is more important than previously expected and that these tissues do not depend on uptake only. Indeed, AGAT and GAMT enzymes are expressed in human skeletal muscle<sup>37</sup> and heart<sup>52 53</sup> and endogenous creatine synthesis has been found in the Müller glial cells of the rat retina.<sup>54</sup> Creatine has been found present in skeletal muscle of two CRTR-D patients by <sup>1</sup>H- and <sup>31</sup>P- MRS<sup>36</sup> and muscle biopsy,<sup>55</sup> respectively. This is in contrast with the undetectable and significantly decreased creatine in the skeletal muscle and heart respectively of the CRTR-knockout mouse.<sup>56</sup> This difference might be explained by species differences in tissue expression of AGAT.<sup>37</sup> CRTR-knockout mice also have significantly decreased serum creatine while plasma creatine in CRTR-D patients was normal. CRTR-knockout mice might be unable to compensate decreased reabsorption of creatine in the kidneys by increasing creatine synthesis.

The brain also synthesises creatine<sup>57</sup> while uptake from the periphery at the blood–brain barrier is limited.<sup>57 58</sup> Cerebral creatine deficiency in CRTR-D has been explained by the observation that GAMT is mainly expressed in glial cells and neurones might rely on CRTR to uptake creatine.<sup>59</sup> Others found that AGAT and GAMT are expressed in all brain cell types but rarely coexpress so that intermediate GAA must be transported between AGAT and GAMT-containing cells via CRTR to insure creatine synthesis.<sup>57</sup> In this model, GAA accumulation would be expected in CRTR-D as in GAMT deficiency. Although slightly elevated cerebral GAA was reported in one CRTR-D patient,<sup>60</sup> this is usually not observed.<sup>44</sup> Furthermore, in this cohort we found normal or only slightly elevated GAA of 0.05–0.44  $\mu$ M in CSF while CSF GAA is strongly elevated to 14–15  $\mu$ M in GAMT deficiency.<sup>43</sup> Recent studies confirmed that neurones and astrocytes are able to synthesise creatine but showed that their intracellular creatine levels depend in vitro far more on uptake than de novo synthesis.<sup>61</sup>

Remarkably, we found normal to elevated creatine level in CSF of CRTR-D patients. In contrast, CSF creatine levels are extremely low in patients with GAMT deficiency, consistent with severely reduced cerebral creatine levels. We hypothesise that the brain synthesises creatine but that in CRTR-D creatine is lost in CSF due to reuptake failure and that the cerebral creatine deficiency derives from defective creatine recycling.<sup>62</sup> Mouse models of neurotransmitter transporter defects confirm the importance of reuptake for maintenance of intracellular neurotransmitter stores.<sup>63</sup> This supports a role of creatine as a neuromodulator as previously suggested.<sup>38</sup>

Urinary excretion of specific organic acids suggesting mitochondrial dysfunction was reported in two CRTR-D patients in this cohort. Reduction of one or more respiratory chain enzyme complex activities were reported in a CRTR-D patient with a contiguous gene deletion and in three patients with either AGAT or

GAMT deficiency.<sup>12 64 65</sup> These findings suggest that creatine deficiency might also affect mitochondrial energy metabolism.

### Diagnosis

All patients had an increased urinary Cr to Crn ratio compared with age-related reference values<sup>43</sup> suggesting a 100% sensitive test to detect males with CRTR-D. The ratio was also increased in all patients initially diagnosed by MRS or molecular genetic screening. However, healthy controls can have elevated Cr to Crn ratio and false positives are regularly detected.<sup>5</sup> A repeat morning urine sample collected after an one-day meat and fish free diet increases the test specificity.<sup>5</sup> Additional molecular genetic testing of the *SLC6A8* gene is necessary to confirm the diagnosis. The urinary Cr to Crn ratio has become a widely available screening test and is in many metabolic laboratories part of the metabolic screening in ID.

<sup>1</sup>H-MRS is also a very sensitive screening method but unavailable in many centres and requires general anaesthesia. Further biochemical or molecular genetic tests are necessary to differentiate CRTR-D from creatine synthesis defects.

With the rapid development of next generation sequencing, molecular genetic screening for CRTR-D will become more common. In case of a novel unclassified variant, the diagnosis in the patient should be confirmed by creatine uptake studies in patient fibroblasts or by proving the pathogenicity of the variant. Missense variants can be classified by creatine uptake studies in CRTR deficient fibroblasts after in vitro overexpression of the mutant allele.<sup>40 41</sup> Neutral or intronic variants should be studied by bioinformatic analysis and mRNA analysis.<sup>39</sup> The recommended diagnostic workup is shown in figure 5.

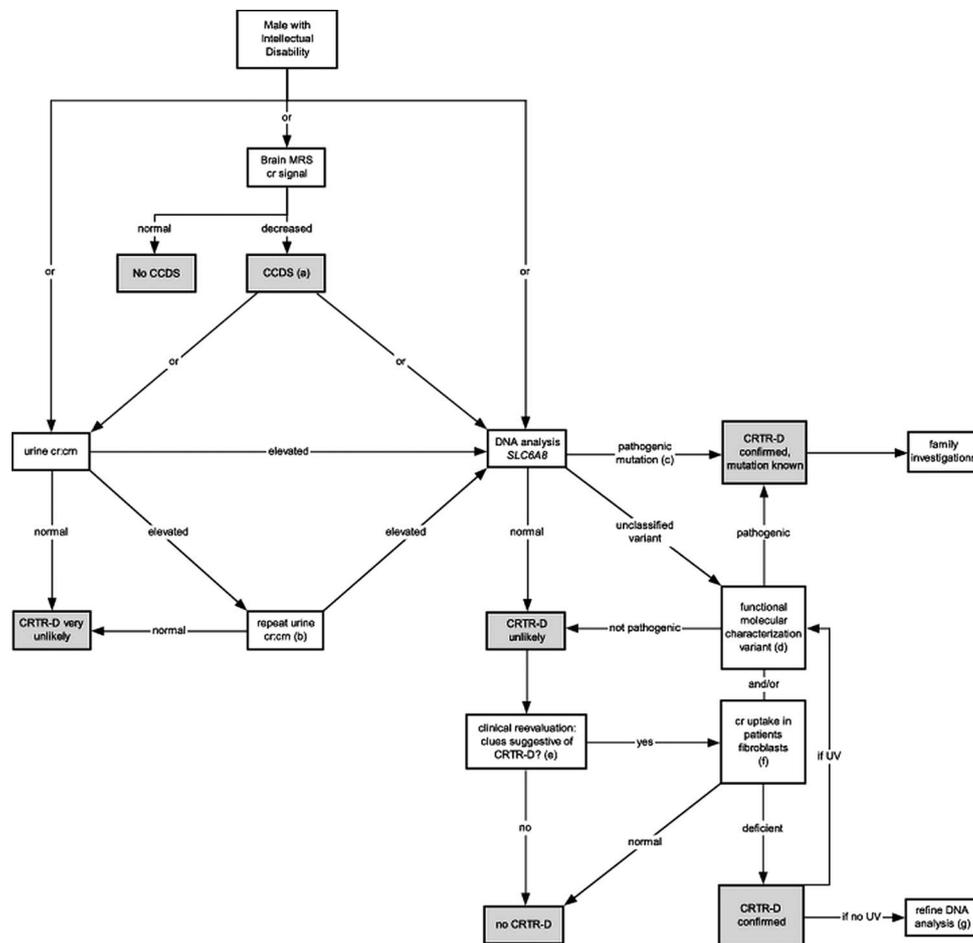
### Genetics

Missense mutations and 3 bp deletions are most common. Two hot spot mutations c.1222\_1224del; p.(Phe408del) and c.1006\_1008del; p.(Asn336del) accounted together for 14% of the families. Notably, four missense mutations, c.1271G>A; p.(Gly424Asp), c.1661C>T; p.(Pro544Leu), c.1699T>C; p.(Ser567Pro) and c.1190C>T; p.(Pro397Leu), were shown to have residual transporter activity.

Mutations occurred de novo in 30% of the index patients and the recurrence risk in further pregnancies of their mothers might be expected to be low. However, we found somatic mosaicism in a relatively high percentage (7%) of mothers including a mother with two affected sons.<sup>66</sup> We warn that low levels of somatic mosaicism and germline mosaicism cannot be excluded with DNA sequencing and that prenatal diagnosis in further pregnancies should always be offered.

### Genotype–phenotype correlation

Missense mutations with residual transporter activity in transfection studies, present in nine patients in this cohort, might be associated with a milder and more variable phenotype. Urinary Cr to Crn ratios in these patients were significantly lower and a remarkably mild ID was found in one family in two of three affected brothers<sup>20</sup> while the third brother had a more typical moderate ID, illustrating variable presentation of the same mutation. Also, one patient with a missense mutation with residual activity had severe refractory epilepsy<sup>17</sup> while three other patients with the same mutation did not have epilepsy. In one patient, creatine uptake in cultured skin fibroblasts was very low despite a consistent residual activity of the missense mutation in the transfection studies. The results of uptake studies in patient fibroblasts are more variable and possibly influenced by other patient factors.



**Figure 5** Diagnostic workup for creatine transporter deficiency (CRTR-D) in males. Urinary Cr to Crn ratio and creatine measured by MR spectroscopy (MRS) are not reliable in females and screening by DNA analysis of *SLC6A8* is recommended.<sup>63</sup> <sup>a</sup> Cerebral creatine deficiency syndromes (CCDS) includes arginine:glycine amidinotransferase deficiency (AGAT-D) and guanidinoacetate methyltransferase deficiency (GAMT-D) besides CRTR-D. Further evaluation should include biochemical or DNA analysis for AGAT-D and GAMT-D. <sup>b</sup> False positives occur; if the ratio is only mildly elevated and there is no strong suspicion of a CRTR-D, repeat the test in a morning sample after a diet devoid of meat and fish. <sup>c</sup> A mutation is considered to be pathogenic if it: (1) is a nonsense mutation, (2) is a (3 bp) deletion, (3) causes a frameshift, (4) causes aberrant splicing or (5) has been proven to be pathogenic, previously. <sup>d</sup> If missense variant, test for restoration of creatine uptake in CRTR deficient fibroblasts after transfection with *SLC6A8* cDNA in which the variant has been introduced by site-directed mutagenesis.<sup>40–41</sup> If neutral variant or intron variance sequence (IVS), perform mRNA analysis if bioinformatic analysis points to a splice site effect.<sup>39</sup> <sup>e</sup> If elevated urinary Cr to Crn, repeat urine analysis. If repeatedly elevated urinary Cr to Crn, continue with brain MRS or creatine uptake in fibroblasts. If decreased brain MRS Cr signal and AGAT and GAMT deficiency are excluded, continue with creatine uptake in fibroblasts. <sup>d+f</sup> If DNA analysis was the first test, perform clinical evaluation (urine Cr to Crn, possibly MRS). <sup>g</sup> The diagnosis is confirmed in the patient but additional molecular analysis is necessary to identify the pathogenic mutation. UV, unclassified variant.

The results of these uptake studies should therefore not be used as predictors of residual CRTR activity and prognosis.

A severe phenotype has been reported in three patients with multi-exon deletions of the *SLC6A8* gene.<sup>12–31</sup> We now know that the deletion included the neighbouring *BCAP31* gene in two patients but in the third patient the breakpoint was located in the non-coding region between the *SLC6A8* and *BCAP31* gene. The severe phenotype might be caused by complete loss of the *SLC6A8* gene. However, a patient with exons 5–12 deletion and patients with frameshift, splice-error or nonsense mutations in the *SLC6A8* gene did not present with the similar severe phenotype. The non-coding region between *SLC6A8* and *BCAP31* might contain a regulatory element, causing the severe phenotype in patients with deletions extending beyond the 3' end of *SLC6A8*.

### Development of treatment

There is no actual treatment for CRTR-D. The effectiveness of L-arginine and glycine supplementation has not been

proven.<sup>21–42–44</sup> Cyclocreatine treatment in a brain-specific *SLC6A8* knockout mouse showed promising results<sup>67</sup> and warrants further studies. Awareness of the phenotype and phenotype-genotype correlation, as presented in our study, is important in the evaluation of future treatment protocols. Evaluation of the biochemical phenotype revealed high creatine in CSF suggesting a defect in reuptake of creatine in the neurons.<sup>62</sup> This might direct future treatment development.

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